INHIBITION OF LECTIN-INDUCED MITOGENIC RESPONSE OF MURINE LYMPHOID CELLS BY THE CHINESE DRUG TIANHUAFEN

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LIST OF ABBREVIATIONS

AAG	:	alpha$_1$-acid-glycoprotein
ALS	:	antilymphocytic serum
α-MM:	:	α-methylmannoside
CMI	:	cell-mediated immunity
Con A	:	concanavalin A
DH	:	delayed hypersensitivity
GVH	:	graft versus host
HCG	:	human chorionic gonadotropin
^{3}H-Leu	:	tritiated leucine
^{3}H-TdR	:	tritiated thymidine
^{3}H-UdR	:	tritiated uridine
IF	:	interferon
Ig	:	immunoglobulin
i.p.	:	intraperitoneal
i.u.	:	international unit
i.v.	:	intravenous
LPS	:	lipopolysaccharide
min	:	minute
MLR	:	mixed lymphocyte reaction
P6	:	protein 6
PBL	:	peripheral blood lymphocyte
PFC	:	plaque forming cell
PPD	:	purified protein derivative
PWM	:	pokeweed mitogen
RIT	:	rosette inhibition titre
s.c.	:	subcutaneous
SRBC	:	sheep red blood cell
TCS	:	Trichosanthin

ABSTRACT

An immunosuppressive protein has been isolated and purified from the Chinese drug, Tianhuafen (*Trichosanthes kirilowii*) by fractional acetone precipitation, ammonium sulfate precipitation and CM-Sepharose chromatography. The purified protein was shown to be homogeneous by immunoelectrophoresis, SDS-gel electrophoresis and gel filtration and has a molecular weight of 24,000. The amino acid composition of the protein was determined and no cysteine could be detected. The immunosuppressive protein was identified to be trichosanthin (TCS), a known abortifacient protein obtained from the same source, by comparison of molecular weight and amino acid composition. The conclusion that the immunosuppressive protein is TCS was further supported by its mid-term abortifacient activity in mice.

Suppressive effects of TCS on cell-mediated immune responses of mice were established by both *in vitro* and *in vivo* studies. At the dose of 100μg/ml, TCS abrogated completely the following *in vitro* immune reactions: Con A- and LPS-induced transformation by lymphocytes isolated from spleen, lymph nodes and thymus of CBA mice; and the one-way mixed lymphocyte reaction of CBA and WHT mouse splenocyte. However, the increase in glucose uptake, lactate production and rate of oxygen consumption in con A-stimulated lymphocyte blastogenesis was only partially suppressed at the same concentration of TCS. *In vivo* administration of TCS

(0.2 mg/25 g body weight) in mice resulted in a reduction in the blastogenic responses of splenocyte to con A and LPS. At the same dose, TCS partially inhibits the delayed hypersensitivity reactions of WHT and CBA mice to SRBC.

The mechanism of inhibition was studied in con A-induced splenic lymphocyte transformation. The observations that cell agglutination mediated by con A as well as binding of ^{125}I-con A to the cells were not affected by the presence of inhibitory concentration of TCS suggested TCS did not interfere with binding of con A to the cell per se. TCS added after 24 or more hours of con A prestimulation caused no inhibition on the blastogenic response, indicating TCS could not exert the inhibitory effect on cells already committed to proliferation. The preliminary studies suggested that the inhibition by TCS is likely to occur following binding of the mitogen but before the cells are committed to cellular division and that the plasma membrane may be the site of action.

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Chapter I : INTRODUCTION

1.1 The Immune System

1.1.1 The organization of the immune system

The immune system defends an organism against foreign, non-self materials, be they malicious or benign. This active defensive role is executed by various types of cells and their secretory products (J.F. Bach, 1976). The cells involved are: lymphocytes, natural killer cells, macrophages, mast cells, basophils, eosinophils, and complement producing liver cells. Among these, lymphocytes play the most important part. The central importance of lymphocytes for immune responses was established largely by the work of Gowans (1959 and 1964). He first showed in 1959 that it was possible to transfer immunocompetence and even immunologic memory to irradiated animals by injecting purified lymphocytes. He further demonstrated that removal of large number of lymphocytes depressed antibody production, and that readministration of thoracic duct lymphocytes restored the immunocompetence of irradiated rats. These findings provide definitive evidence of the exclusive role of lymphocytes in specific immunity.

Lymphocytes are divided into two major categories, the B and the T lymphocytes. (The B refers to the bursa of Fabricius of birds or to the bone marrow in mammals. The T refers to the thymus.) B lymphocytes derive from bone marrow stem cells through a process of antigen-independent maturation that takes place in birds in the bursa of Fabricius. No primary lymphocyte organ for B cell differentiation has been identified in mammals, although part of the process takes

place in bone marrow. The T lymphocytes derive in all species from the thymus. B and T lymphocytes are not homogeneous and include various subsets of cells. There is a third category of lymphocyte that does not show the classical characteristics of T and B cells. These have received the ill-defined name of null or K cells (Golub, 1977). Null cells may include stem cells and certain types of cytolytic cells. Their definite identification as a third lymphocyte population has not been fully established.

The lymphocytes are in a highly dynamic state with a large number of them recirculating as individual cells in blood and lymph fluids. The lymphocytes are the main cells of the lymphoid tissues thymus, lymph nodes, and spleen and an important constituent of some non-lymphoid tissues, forming the diffuse lymphoid component, for example, of the respiratory and gastrointestinal tract which is dominated by T-lymphocytes. Thymus is a primary lymphoid organ where mature T cells are generated from bone marrow stem cells. Lymph nodes and spleen formed by mature B and T cells as well as antigen-trapping cells such as macrophages are secondary lymphoid organs. It is in the secondary lymphoid organs where antigen is concentrated and where the specific immune reactions are generated.

Under the light microscope, unstimulated lymphocytes are morphologically alike. They appear as small round cells (diameter 7-12 μm) with a large round nucleus and a thin rim of cytoplasm. Recent examination of human lymphocytes by scanning electron microscopy has revealed a clear

morphological dichotomy between the microvilli bearing B cell and the smooth T cell (Barrett 1978). Functionally, lymphocytes can also be divided into two subclasses, T and B. T cells are responsible for eliciting cell-mediated immunity (CMI), while B cells, for humoral immunity.

1.1.2 Humoral and cell-mediated immunity

The immune response comprises all the complex series of events which occur as a result of the specific interaction of an immunocompetent cell with an antigen. There are two broad categories of immune responses :

1) humoral immunity, the production by stimulated lymphocytes of antibodies which travel in the bloodstream and act on antigenic particle or molecules at some distance from their source of origin; and

2) cellular (or cell mediated) immunity, a process whereby immunologically active T lymphocytes actually make contact with foreign antigen and exert a local, direct action.

Cellular immunity is responsible for reactions such as delayed hypersensitivity (DH) reactions, mixed lymphocyte reaction (MLR), foreign graft rejection, and resistance to some kind of infection (Miller, 1978). In the present study, the effect of Trichosanthin, a protein isolated from the Chinese herb, Tianhuafen (*Trichosanthes kirilowii*), on some cell-mediated reactions, including lymphocyte blastogenic responses to mitogens and allogeneic lymphocytes (MLR) and delayed hypersensitvity (DH) to sheep red blood cells (SRBC) was investigated.

1.2 Lymphocyte Transformation

Unstimulated lymphocytes are quiescent and remain in Go phase outside the mitotic cycle (Berger, 1979). Following activation with suitable stimuli, they undergo differentiation and transformation into blast cells, culminating in DNA synthesis and mitosis. Lymphocyte transformation can be induced by at least four different types of stimulants (Cooper, 1973): 1) the non-specific mitogen, including plant lectins and certain microbial products, 2) the specific antigens that the lymphocyte has been sensitized, 3) allogeneic lymphocytes and 4) the products of stimulated lymphocytes (lymphokines). There are a number of reviews on lymphocyte transformation induced by various mitogens (Ling and Kay, 1975). The present discussion will be focused on the mitogenic activation of lymphocytes. For other types of stimulation, readers are referred to Ling and Kay (1975).

Lymphocyte stimulants can be classified as specific or non-specific depending on whether or not response to the stimulus requires prior exposure of the cell to the stimulus. Mitogenic stimulation is non-specific while antigenic stimulation is specific. Both mitogenic and antigenic stimulation bring about similar sequences of events, differing in the kinetics and magnitude of their responses. Mitogens induce an earlier and greater peak response than antigens (Cunningham *et al.*, 1976).

1.2.1 The phenomenon

A number of morphological and metabolic changes take

place during lymphocyte transformation (Loeb, 1974). Unstimulated lymphocytes are characterized by a large eccentric nucleus surrounded by a thin rim of cytoplasm; these cells show a relatively low rate of synthesis of protein, RNA and DNA. Following stimulation with mitogen, lymphocytes undergo dramatic alteration. The cells enlarge and develop a prominent cytoplasmic component filled with rough endoplasmic reticulum for active protein synthesis. The synthesis of RNA and DNA is also increased. The stimulated lymphocytes assume the appearance of lymphoblasts and may undergo mitotic division. Lymphocyte transformation can be scored morphologically by counting the number of blast cells among the population of lymphocytes or by measuring the incorporation of radiolabeled precursors into newly synthesized protein or DNA.

Lymphocyte transformation is initiated by the binding of the mitogen to the outer cell membrane where specific receptor molecules recognizing the stimulant are present (Greaves and Bauminger, 1972). The transmission of the binding signal to the inner cell machinery is brought about by a variety of biochemical events that take place subsequently.

Molecular changes that occur during lymphocyte transformation may be arbitrarily divided into four time periods (Loeb, 1974). Early events (within 2 h) are characterized by alterations in membrane structure and function, as well as by changes in nuclear metabolism. In relation to the membrane, marked increases in the uptake of a large spectrum of cations and metabolites are observed. The nuclear changes which occur are suggestive of alterations

in both structrual and regulatory chromosomal proteins. The intermediate events (2 to 14 h) centre on increases in RNA synthesis and the assembly of ribosomes for protein synthesis. The late events (15 to 72 h) focus on DNA replication and increases in the enzyme activities associated with this process. After the peak of DNA synthesis and cell division, an ebbing period when all molecular activites return to prestimulation level ensues.

1.2.2 The mitogens

Antigens are not the only agents which can trigger lymphocytes. A number of substances, known as mitogens, have the capacity to interact with cells of the immune system resulting in the mitosis of the blast cells. In 1960, Nowell first discovered the plant protein, phytohemagglutinin (PHA) as a mitogen (Nowell 1960). Since then, a variety of mitogens have been isolated from various sources, including plants (Barker, 1969; Parker et al., 1969 and Curtain and Simons, 1972)and microorganisms (Rodey et al., 1972 and Kreger et al., 1972). Some degree of lymphocyte activation has also been produced by periodate (Novogrodsky and Katchalski, 1972), zinc ions (Cunningham et al., 1980), mercury ions (Caron et al. 1970), and sequential neuraminidase and galactose oxidase treatments (Novogrodski and Katchalski, 1973).

The most well studied mitogens are the plant lectins. Farnes et al. (1964) found that pokeweed mitogen (PWM) isolated from Phytolacca americana was mitogenic for both T and B lymphocytes in the mouse, but was only weakly

erythro-agglutinating. PHA isolated from Phaseolus vulgaris was first reported by Nowell (1960) to be mitogenic. Allen et al. (1969) separated crude PHA into two different fractions. One of these fractions termed PHA-L was essentially mitogenic, while the other, PHA-H, was a potent hemagglutinin. The exact concentrations of PHA required for lymphocyte activation depend very much on the precise experimental conditions. For human lymphocytes in medium containing 10% serum, the optimal PHA concentration is 1 - 5 μg/ml (Ling and Kay, 1975).

Concanavalin A (con A) isolated from Canavalia ensiformis is the best characterized lectin. It was first obtained in a crystalline state by Sumner (1919). Its hemagglutinating property was reported (Powell and Leon, 1970) and specificity investigated in detail (Sharon and Lis, 1972). Con A agglutinates erythrocytes, although less powerful than PHA, by binding to saccharide residues on their surfaces. It binds sepcifically to α-glucopyranosides and α-D-mannopyranosides, and to polysaccharides or glycoproteins containing such residues. Nilsson and Waxdal (1976) isolated and identified the major con A-binding glycoproteins from murine spleen cells, thymocytes, purified thymus-derived lymphocytes and from the spleen cell of congenitally athymic (nude) mice. Its amino acid sequence (Edelman et al., 1972) and tertiary structure (Cunningham et al., 1974) have been determined. Characteristics of the mitogenic activity of con A were studied extensively by Andersson et al. (1972a). They found that when T cells were removed from spleen cell suspension, the mitogenic effect of con A was markedly

reduced. Spleen cells from mice depleted of T cells by thymectomy and lethal irradiation and then repopulated with B cells did not respond to con A. These results established the selectivity of con A for T lymphocytes.

The above described plant lectins selectively stimulate T cells (con A and PHA) or stimulates both T and B cells (PWM). The isolation of a B cell specific mitogen was first reported by Peavy et al. (1973). They discovered that lipopolysaccharides (LPS) with endotoxin activity produced by E. coli were mitogenic for mouse and rat spleen cells with selectivity for B lymphocytes, but had little effect on human or rabbit blood lymphocytes. Endotoxins from Salmonellae, e. g. some smooth and rough strains of Salmonella typhimurium, were also mitogenic for mouse spleen cells. Lipopolysaccharides from Gram-negative bacteria consist of a polysaccharide and a lipid component termed lipid A. Peavy et al. (1973) found that the lipid portion of the molecule is essential for mitogenic activity, while the isolated polysaccharide part is inactive.

1.2.3 The mechanisms of activation

Lectin-induced lymphocyte transformation has been widely used as a model for cell activation and proliferation. The activation can be divided into three stages : the binding stage, the activation stage and the effectuation stage. Numerous research has delineated quite substantially events occurring during the binding and effectuation stages. However, how binding of mitogens activates the cellular machinery

remains obscure.

1.2.3.1 binding

Lymphocyte stimulation is initiated by the attachment of the stimulant to the outer cell membrane. Simons et al. (1968) showed that maximal stimulation occurs when approximately 10^6 molecules of PHA are attached to each lymphocyte. Similarly, Betel and von den Berg (1972) calculated that 2.5×10^5 molecules of con A bound per lymphocyte resulted in maximal stimulation. That this binding is non-covalent and is a prerequisite to activation are indicated by experiments of Andersson et al.(1972a) in which removal of con A from the cell surface by a specific inhibitor (α-methyl-D-mannoside, α-MM) abolished the mitogenic response.

Greaves and Bauminger (1972) reported that mitogens covalently attached to Sepharose beads were capable of stimulating mouse lymphocytes. This concept of a mitogen not entering the cell is supported by experiments of Andersson et al. (1972b) in which lymphocytes were stimulated by con A covalently linked to a plastic support.

Gunther and Wang (1974) investigated the kinetics of proliferation in con A stimulated mouse spleen cells. The competitive inhibitor α-MM was added to cultures at various times after con A stimulation. When the inhibitor was added after cells had been exposed to con A for 24 h or more, no inhibition was observed. They concluded that once the cell is committed to transformation, the presence of con A is no longer required.

1.2.3.2 Changes during effectuation stage

The sequence of synthetic activities subsequent to lymphocyte activation would naturally necessitate an ample supply of synthetic precursors, energy and the enzymes involved in such syntheses.

Increased uptake of α-aminoisobutyric acid, 3-O-methyl-glucose, K^+ and Ca^{++} (Loeb, 1974) have been reported. A large number of enzyme activities were reported to have increased (Loeb et al.,1970 and Rabinowitz et al.,1970). Amongst these enzymes, the most dramatic increase in enzyme activity was found with DNA polymerase, ranging from 30 to 200 folds (Loeb et al.,1968). Addition of PHA (Parkes and Howell, 1975) and con A (Wang et al.,1976a)to lymphocyte cultures were reported to enhance glucose consumption, lactate production and activities of glycolytic enzymes. The oxygen consumption of PHA stimulated lymphocytes has been reported to be increased by some workers (Pachman, 1967; Roos and Loos, 1973), while others have been unable to demonstrate any change in respiration(Polgar et al.,1968; Culvenor and Weidemann, 1976). This discrepancy may be related to the measurements being taken at different times after stimulation.

1.2.3.3 Signal transmission

How the signal of mitogen binding on the cell exterior is transmitted to the cell interior and

subsequently results in activation of the cell machinery remains largely unsolved. During lymphocyte activation, diverse biochemical pathways in different parts of the cell are affected more or less simultaneously beginning quite early in the response. The involvement of a diffusible intracellular messenger with broad flexibility as a regulatory agent would logically be required. Candidates having this pleiotypic quality are the cyclic nucleotides and a variety of critical intracellular ions, e.g. Ca^{++} and K^{+}.

Wedner et al.(1975) reviewed the role of cAMP in lymphocyte activation. They confirmed that cAMP played a part at least in some of the very early biochemical alterations in activated lymphocytes but was doubtful about reports of cGMP having positive influence on lymphocyte activation. The part played by cGMP was reviewed by Hadden et al.(1975). They claimed strongly for a positive regulatory role of cGMP in induction of lymphocyte proliferation. The validity of the speculations of these two groups of workers remains controversial. Other workers have discussed the roles of phospholipids (Resch and Ferber, 1975), divalent cations (Maino et al.,1975), microtubules (Oliver, 1975) and cation transport (Kaplan and Quastel, 1975). Whether one of these factors is responsible for, or some or all of them interplay to effect the activation awaits further investigations.

1.3 Suppression of Lymphocyte Transformation

Soon after the discovery by Nowell (1960) of the mitogenic property of PHA on lymphocytes, a class of substances was found to possess antagonistic effects on this mitogenic activity. The corticosteroid, prednisolone, was reported independently by Nowell (1961) and Tormey et al. (1961) to inhibit PHA-induced DNA and RNA syntheses in human lymphocytes. Since then, a wide spectrum of agents having inhibitory effects on mitogen induced lymphocyte transformation has been reported. These agents are classified here into three categories: 1) organic and inorganic small molecules, 2) heterogeneous factors secreted by lymphoid cells and found in serum and 3) peptides and proteins. Many of these agents suppress other immune responses as well. In fact, the ability to suppress lymphocyte transformation is one of the many screening tests for immunosuppressive agents (see table 1.1, Bach, 1975).

In the following discussion, immunosuppressive agents that are protein in nature will be emphasized, as they are more related to the present investigation. There are good reviews on action of small molecules and heterogeneous factors (Bach, 1975 and Spreafico and Anaclerio, 1977), which will hereby be described only briefly.

TABLE 1.1 Screening tests for immunosuppressive agents

A. In vitro tests

(performed directly on lymphocytes from animals treated by the drug or on normal lymphocytes with the original drug or with serum of drug treated animals)

1. Inhibition of spontaneous rosette formation with sheep red cells by mouse and human cells
2. Inhibition of mixed lymphocyte reaction
3. Inhibition of in vitro response to phytohemagglutinin and concanavalin A
4. Inhibition of the primary in vitro immune response to SRBC (Mishell and Dutton's technique)

B. In vivo tests

1. Antibody response to sheep red blood cells
   - -humoral antibodies: agglutinins, hemolysins
   - -cellular responses
     - B cell: ϑ-negative, azathioprine-resistant RFC*
     - T cell: ϑ-positive, azathioprine-sensitive RFC
2. Antibody response to
   - bovine serum albumin (thymus-dependent antigen)
   - polyvinyl pyrrolidone (thymus-independent antigen)
3. Cell-mediated immunity
   - skin allografts crossing or not the H2 barrier

* RFC : Rosette Forming Cells

1.3.1 Organic and inorganic small molecules

The non-protein inhibitors of lymphocyte transformation include the clinically used immunosuppressive agents: the thiopurines (6-mercaptopurine and azathioprine), the alkylating agents (cyclophosphamide), the glucocorticoids (prednisolone) and the folic acid antagonist methotrexate. Most of these reagents exert their immunosuppressive effects by virtue of their cytotoxicity on the lymphoid system (Floersheim, 1978). Other less well-studied agents include isoptin (a calcium antagonist, Blitstein-Willinger and Diamantstein, 1978), amphotericin B (an antibiotic, Ferrante and Thong, 1979), quinine (an antimalarial compound, Thong and Ferrante, 1978), morphine (Ho and Leung, 1979), egg lecithin (Ng et al., 1978), a bovine glycosphingolipid (Lengle et al., 1979) and the antibiotics actinomycin and chloramphenicol (Minich, 1973).

1.3.2 Heterogeneous, chemically undefined immunosuppressive agents

In contrast to the above category of chemically well-defined agents, included in this group are inhibitory activities associated with sera of patients suffering from particular diseases, supernatants of cultures of special cell types or the cells themselves. Their active principles have not been isolated, characterized, or identified.

1.3.2.1 Serum

Leukemic sera isolated from children with acute lymphoblastic leukemia have been reported to inhibit PHA-induced lymphocyte transformation (Humphrey et al.,1974). This inhibition was assumed to be due to binding of PHA by components of leukemic serum because the inhibition could be overcome by increasing the PHA concentration. Anti-lymphocyte sera (ALS), which are heterologous sera prepared by the injection of lymphocytes into a heterologous animal (Revillard, 1970), have been shown to inhibit lymphostimulation induced by mitogens and antigens (Scharwz, 1970). Levey and Medawar (1966) postulated that this suppressive effect was due to inactivation of ALS coated lymphocytes which are unable to recognize antigens through their surface receptors.

1.3.2.2 Cell products

Culture fluids from peritoneal exudate cells rich in macrophages inhibited the spontaneous proliferation of several tumour cell lines and the con A- or bacterial toxin-induced mitogenesis (Unanue et al.,1975). Ranney (1975) observed that a dialyzable factor in the culture supernatants of neonatal spleen cells produced a marked reduction in the incorporation of tritiated thymidine (^{3}H-TdR) by stimulated autologous thymocytes. Human lymphotoxins from lectin stimulated peripheral blood lymphocytes (PBL) have also been reported to inhibit the

proliferation of HeLa cells *in vitro* (Wave and Granger, 1979). Olding *et al.* (1977) reported the suppression of maternal lymphocyte proliferation by mitogen-stimulated lymphoid cells from human newborns across a cell-impermeable membrane.

1.3.2.3 Cell-cell interaction

Bassett *et al.* (1977) reported the suppression of stimulation of MLR by newborn splenic lymphocytes in the mouse. Chicken bursal cells, spleen cells from inbred rats were shown to reversibly suppress the incorporation of ^{3}H-TdR when added to proliferating autologous thymocytes (Ranney, 1975). Whether this inhibition was mediated by cell-cell interaction or release of soluble mediators is still unknown.

1.3.3 Peptides and proteins

1.3.3.1 From animal sources

A number of protein molecules having immunoregulatory properties have been isolated from animal sources, including cell extracts, blood and supernatants of cell cultures.

Serum proteins occurring naturally or found only in animals suffering from a particular disease have been reported to possess immunoregulatory effects. Cooperband *et al.* (1972) reported the expression of an immunosuppressive factor from α-globulin containing

fractions of human plasma proteins. This factor has been shown to inhibit PHA-induced lymphocyte transformation. Chiu (1977) observed that α_1-acid glycoprotein (AAG), a constituent of normal serum elevated in concentration in the acute phase of inflammation markedly inhibited the proliferative response of human PBL to PHA.

Interferons (IF) are soluble proteins released by lymphocytes, macrophages and a variety of non-lymphoid cells in response to a variety of stimuli. They are characterized by their capacity to inhibit replication of a number of unrelated RNA and DNA viruses. More recently, IF's were shown to depress immune activities, including graft-versus-host reaction, allograft rejection, lymphocyte recognition *in vitro* and *in vivo* of alloantigens, and the primary and secondary anti-sheep red blood cell (SRBC) responses (Spreafico and Anaclerio, 1977).

A chalone is a tissue-specific, non-species-specific endogenous mitotic inhibitor that is protein in nature. It is present in and synthesized by the tissue on which it exerts an antiproliferative activity through a noncytotoxic mechanism. Various *in vivo* and *in vitro* immune reactivities have been shown to be inhibited by chalones (Houck, 1976). These activities include a decrease in PHA reactivity with a block in migration inhibitory factor release and depressions of anti-SRBC responsiveness, allograft rejection, and GVH activity. The ^{3}H-TdR uptake, mitotic index and the cell number in mitogen-stimulated normal human lymphocytes were

also depressed by crude lymphoid chalone extracts. However, lymphoid chalones were reported to be effective only in suppressing *in vitro* immune responses (Hiestand *et al.*, 1977) but has no effect on *in vivo* immune responses (Borel *et al.*, 1978).

Amongst serum proteins having immunosuppressive properties, alpha-fetoprotein (α-FP) has been studied in detail. The synthesis of α-FP is associated with the processes of normal, restorative and malignant growth as reflected by its synthesis by the fetal liver during embryogenesis, its expression during hepatic regeneration after various forms of injury, and its appearance in the sera of certain tumour bearing hosts, most notably in primary hepatocellular carcinomas (reviewed by Murgita and Tomasi, 1975). These same authors first proposed an immunoregulatory function of α-FP basing on their demonstration that α-FP, either in the isolated form or as it occurred in amniotic fluid, exerted a non-cytotoxic immunosuppressive effect on the primary and secondary antibody response. Using mouse amniotic fluid, they showed that α-FP suppressed certain T-cell dependent functions such as allogeneic lymphocyte stimulation in the one-way MLR and mitogenic effects of PHA, con A and LPS on mouse spleen cells. Dose response experiments showed that the suppressive effect of α-FP on the response to mitogens and to allogeneic cells diminished linearly with inhibitory activity still evident at 1 μg/ml. Goeken and Thompson

(1977) reported that the immunosuppressive activity of ∝-FP may depend both on its source and the procedure by which it was isolated. Sheppard et al. (1977) and Sell et al. (1977) expressed a skeptical view on the immunoregulatory property of ∝-FP basing on their own experimental results. They found that ∝-FP by itself was mitogenic for mouse lymphoid cells. Both normal and ∝-FP-rich sera inhibited con A, PHA and MLR proliferative responses of lymphocyte cultures in vitro equally well, in spite of a difference in ∝-FP concentration of up to 100,000 fold. Alpha-fetoprotein-rich sera had no effect on the antibody response of the rat in vivo. Purified ∝-FP from tumour sera was not inhibitory at 100 μg/ml for con A, PHA or MLR reactions and only inhibited PHA stimulation at medium concentrations greater than 1,000 μg/ml. They were unable to verify that ∝-FP was generally immunosuppressive in the rat and mouse. Experiments of Hassoux et al. (1978) also support the lack of suppressive activities of ∝-FP purified from crude mouse amniotic fluid which was inhibitory on mitogenic blastogenesis and MLR.

Human chorionic gonadotropin (HCG) is a glycoprotein hormone which is produced in large amounts by the trophoblast. It is readily detectable in maternal serum starting from the first two weeks of pregnancy, and is essential for maintenance of pregnancy. Its immunoregulatory role has been studied extensively in view of the maternal tolerance towards an antigenically foreign foetus.

Adock et al. (1973) reported the reversible and non-cytotoxic inhibitory effect of HCG on PHA-induced transformation in human lymphocytes. Morton et al. (1974) found the central suppression of T-cells during pregnancy suggested by the increased rosette inhibition titre (RIT) found in pregnant over non-pregnant mice. The RIT rose early in pregnancy and fell to within the normal range about 7 days before delivery. Han (1975) described an inhibitory effect of HCG on delayed hypersensitivity (DH) skin test responses in guinea pigs given 4000 i.u. of HCG intraperitoneally (i.p.) 1-7 days prior to skin testing. Inhibition of lymphocyte response to PHA and purified protein derivative (PPD) in HCG-treated animals was also observed. Suppression of MLR reaction in human was reported by Teasdale et al. (1975) and Beling and Weksler (1974). Carter (1976) described the diminished cell-mediated immunity as measured by a contact allergic reaction to picryl chloride. Slater et al. (1977) observed that HCG modified the mortality of GVH disease in mice resulting from immunosuppression with cyclophosphamide and challenge with spleen cells. The inhibitory effects of HCG on mitogenic response, induction of antibody formation and expression of plaque-forming cells (PFC) were observed by Hammarstroem et al. (1979). In fact, they even observed that one of the HCG preparations used showed a dose-dependent stimulation on human lymphocytes.

However, Caldwell et al. (1975) reported that purification of HCG removed its immunosuppressive effects at the same time that its biological potency was increased. Maes and Claverie (1977) and Muchmore and Blaese (1977) examined the immunosuppressive properties of crude and purified preparations of HCG. Both groups of workers found the suppressive activity to be associated with the crude preparation, and purification resulted in loss of suppressive activity. They believe that the suppressive principle was a component of the pregnancy urine that contaminated the commercial samples. Whether such a component exists in pregnancy urine remains to be established.

1.3.3.2 From plant sources

The voluminous literature on immunosuppressive agents mainly describes organic molecules or factors and mediators produced by animal tissues. Relatively little information has been gathered about plant extracts having suppressive effects on immune responses.

It has been known for many years that the lectins of Ricinus communis (castor bean) are highly toxic. Recently, several groups have shown that these lectins inhibit the syntheses of protein and DNA by experimental tumour cells (Lin et al., 1971) and rat ascite tumour cells (Onozaki et al., 1972). Kornfeld et al. (1974) observed a marked inhibition of protein and DNA syntheses when the Ricinus phytohemagglutinins were incubated with mouse L1210 leukemic lymphoblasts. Synthesis of DNA in normal human lymphocytes stimulated with mitogens was inhibited similarly.

The cyclic peptide extracted from Fusarium equiseti, cyclomunine, was reported to suppress the in vitro MLR and lymphocyte transformation response of rat and dog lymph node cells and peripheral blood mononuclear cells (Bryan et al., 1980). Amiel et al. (1980) found that cyclomunine prevented the GVH reaction induced by allogeneic spleen cells in mice and prolonged the survival of a rat kidney transplant.

A guanylate cyclase inhibitor was isolated from the balsam pear, Momordica charantia abbreviata (Vesely et al., 1977). The inhibitor was capable of abolishing the guanylate cyclase activity in all rat tissues studied, including the spleen, kidney, heart, liver and other organs of the gut, as well as in carcinogen induced liver cells. The inhibition was specific in so far as adenylate cyclase activity was not affected. The inhibitory activity was acid-stable and heat-labile, had been partially purified (30 fold), and did not appear to be a lipid. Further experiments (Claflin et al., 1978) showed that the extract abolished the in vitro growth of an undifferentiated adenocarcinoma of the rat prostate. The inhibition was not due to a cytotoxic effect of the extract as monitored by trypan blue exclusion.

1.4 Other Cell-Mediated Immune Responses

Cell-mediated immunity (CMI) is T cell mediated immunity transmissible by lymphoid cells and macrophages but not by serum. It depends on close physical contact between specific 'effector cells' produced as a consequence

of immunization with antigen wherever it may be localized (Ford, 1973). Examples are lymphocyte activation, graft rejection and delayed hypersensitivity (DH) reactions.

1.4.1 Mixed lymphocyte reaction

Bain et al., (1964) observed that when leucocytes from the blood of two unrelated individuals were mixed together and cultured for several days, the proportion of lymphoblasts in the mixed cultures was always significantly greater than in the corresponding unmixed cultures. This MLR is simply a special example of lymphocyte activation by cells bearing foreign antigens. MLR is a general phenomenon: it occurs with mixtures of lymphocytes from many and possibly all species and with lymphocytes from various lymphoid tissues (Sorensen, 1972). If one cell population recognizes the other as foreign, it begins to proliferate. This proliferation can be quantitated by the incorporation of radiolabeled precursors into RNA, protein and DNA, as in mitogenic transformation.

The activation is associated with transplantation antigens on the surface of lymphocytes and is normally a two-way reaction, both populations acting as 'stimulator' and 'responder' cells. It is an inborn activity, no prior immunization of one donor with cells of the other being necessary. The reaction appears to arise from direct cell-cell interaction and activation does not occur when the two populations are separated by a millipore membrane (Chapman and Dutton, 1965). The role of macrophages as helper cells is still uncertain (Levis and Robbins, 1970).

Bach and Voynow (1966) developed an improved method for the MLR. One-way stimulation was achieved by treating the cells of one individual with mitomycin C. Treated cells were then rendered tolerant to antigens of the other and could not respond by incorporating ^{3}H-TdR. In this way, treated cells act only as the stimulator, while untreated cells, the responder.

1.4.2 Delayed hypersensitivity

Hypersensitivity reactions are violent immune reactions in the affected individual to a stimulus. There are two main forms of hypersensitivity reaction: the immediate or anti-body mediated type and the delayed or cell-mediated type. The delayed hypersensitivity (DH) reactions represent reactions that appear earliest at least 24 h. after antigenic challenge of a pre-sensitized animal. The tuberculin response is a classical example of DH. During the reaction, the site of antigenic challenge is infiltrated with large numbers of mononuclear cells, mainly lymphocytes, with about 10-20 % macrophages. A possible explanation is that there are some sensitized cells possibly having antibody-like receptors on their surface. These cells interact with antigen and in some as yet unknown way, influence other lymphocytes and phagocytes to migrate to the area.

Delayed hypersensitivity to SRBC in mice was first reported by Nelson and Mildenhall (1967) , who used a

subcutaneous (s.c.) or intraperitoneal (i.p.) sensitization and the foot-pad test. Recently, the development of DH in mice to SRBC was again observed by Miller *et al.* (1973), who used a s.c. sensitization with SRBC in saline. Intraperitoneal sensitization with SRBC in saline also appeared to produce a considerable skin DH in mice (Kerckhaert, 1974). One of the most interesting finding was the demonstration of the ability of mice to develop DH after intravenous (i.v.) sensitization with SRBC in saline (Kettman, 1972), which was the underlying principle in the present study.

Mitsuoka *et al.* (1978) speculated that DH to SRBC was a T-cell dependent phenomenon in both sensitization and effector stages and that it required macrophages for manifestation. They also proved that this reactivity was of the cell-mediated, tuberculin type hypersensitivity. The DH to SRBC showed a distinct tendency to differ with the strain and age of the mice.

1.5 The Tianhuafen Protein

The Chinese drug Tianhuafen is the root tuber of the medicinal plant, *Trichosanthes kirilowii*, of the Cucurbitaceae family. Other parts of the plant, namely, the rind of the fruit and seeds, are also used in medicine as the Chinese drug, Gualou. Recent studies (Yueh and Cheng, 1974 and 1980) on the medical botany of Tianhuafen and Gualou showed that species related to *T. kirilowii*, such as

T. japonica, T. uniflora and T. cucumeroides, are also used as sources of these two Chinese drugs in various regions of China. Among the species used, T. kirilowii is of the highest quality and yields the standard Tianhuafen and Gualou. Tienhuafen and Gualou have been prized as effective remedies for heart diseases, tumors and other ailments. In "Pen Ts'ao Kang Mu", the most cited Chinese pharmacopeia (Li Shih-chen, 1596), Tianhuafen was recorded to have the function of inducing menstruation and to expel fetal membranes.

Based on ethnomedical information and empirical experiences, Tianhuafen has been clinically developed as an effective mid-term abortifacient drug in China in the early 1970's. Scientific studies (Anon., 1976) have confirmed the abortifacient activity in laboratory animals and resulted in the isolation of the active plant protein, Trichosanthin (TCS). TCS was isolated from the crude extract of the fresh root tuber of T. kirilowii and purified by chromatography on CM-Sephadex C-50, and characterized as a basic protein with a molecular weight of approximately 18,000 (Wang et al., 1976b). Later, the Chinese scientists found that TCS contains no cysteine and has a molecular weight of 24,000 (Wang et al. 1979). Purified TCS was found to be effective in inducing mid-term abortion, curing ectopic pregnancy and exerting therapeutic action on choriocarcinoma in woman due to its specific action on syncytiotrophoblast (Hsu et al., 1976; Xiong et al., 1976a). The work done on the reproductive pharmacology of TCS has been reviewed (Anon, 1976). TCS,

being a plant protein, is strongly antigenic and causes allergic reactions in human and laboratory animals. In order to clarify the relationship between the immunogenic action and the abortion induced by TCS, Xiong et al. (1976b) examined the effect of phenargen, an inhibitor of antibody formation and delayed hypersensitivity, on TCS-induced abortion in mice. Their results showed that phenargen inhibited the allergic reaction to the crude extract of T. kirilowii, but did not affect the fetus and abortion still occurred. Thus, the possibility of induction of abortion through immunologic response was eliminated. In fact, the present study discovers and establishes that TCS is by itself an immunosuppressive agent. With purified TCS supplied by the Chinese scientists, Chang and coworkers (Chang et al., 1979; Lau et al., 1980) studied the reproductive physiology of TCS in several laboratory animals. They found that TCS was very effective in inducing mid-term abortion in mouse and rabbit, but was ineffective to terminate pregnancy in rat and hamster even with higher doses. Their most significant finding was that TCS appeared to be more effective and associated with less side effect than prostaglandin $F_{2\alpha}$, an established abortifacient, in rabbit (Chang et al., 1979).

In late 1977, a research program on the bioactive proteins from Chinese herbs was initiated in our laboratory and Tianhuafen was chosen for study on its abortifacient protein, TCS. TCS has been isolated and purified to homogeneity from T. kirilowii and T. cucumeroides by

purification schemes developed in our laboratory (Yeung et al. 1981). The detection and isolation of TCS in T. cucumeroides serve to justify the use of T. cucumeroides as substitute for T. kirilowii in Chinese medicine. A new abortifacient protein different from TCS was isolated from a related Chinese drug, Mubie, Momordica cochinchinesis also of the Cucurbitaceae family (Yeung et al., 1980a). In addition to abortifacient effect, our group explore other biological activities of T. kirilowii. Wong (Wong, 1981; Yeung et al., 1980b) discovered and isolated three galactose-binding lectins, hitherto unreported in the literature, from T. kirilowii. Recently, we (Ng et al.,1980) discovered that protein extract from T. kirilowii abrogated the blastogenic responses mediated by mitogenic lectins such as con A and PHA in mouse splenic lymphocytes. Subsequent work (Yeung et al., 1980c) resulted in the isolation of the immunosuppressive protein which was identified as TCS. The present study concentrated on the immunosuppressive effects of TCS on cell-mediated immunity in mouse. The effects of TCS on lymphocyte transformation induced by T and B cell mitogens, on mixed lymphocyte reaction, on delayed hypersensitivity to SRBC, on glycolysis of con A-induced lymphocyte transformation as well as the kinetics of the inhibitory effect on con A-induced lymphocyte transformation were studied and described in this thesis.

Chapter II: MATERIALS AND METHODS

2.1 Animals and Cells

Inbred CBA/H mice were obtained from the University of Hong Kong and bred by brother-sister mating. Mice of age 12 to 20 weeks were used throughout the present study. CBA mice were used in all experiments unless otherwise stated. Inbred WHT mice of age 8 to 20 weeks were used for mixed lymphocyte reaction experiments. Fresh sheep blood was obtained from the Castle Peak substation, Agriculture and Fishery Department. Cells were washed thrice in PBS and adjusted to the appropriate concentration before use. Cells can be kept at $4^{o}C$ for one month without losing the ability to elicit a delayed hypersensitivity response.

2.2 Medium, Buffers and Solutions

All reagents used were of the analytical grade.

2.2.1 Culture medium

The medium used for suspending and culturing splenocytes was RPMI 1640 (KC Biological, Lenexa, Kansas). Ten grams of the powder and 2 grams of $NaHCO_3$ (BDH) were dissolved in 1 liter double

distilled water and then pressure filtered through millipore filter. Each 500 ml of plain medium was supplemented with the following to make complete medium:

i) Fetal Calf Serum (Gibco, Grand Island, N.Y. Cat. #614) heat-inactivated in 56°C water bath for ½ hour; 50 ml.

ii) Penicillin-streptomycin (Gibco), penicillin 10,000 U/ml, streptomycin 10,000 μg/ml; 5 ml.

iii) Mycostatin suspension (Gibco), 10,000 U/ml, 1 ml.

iv) Gentamycin (Schering Corporation), 50 mg/ml; 0.15 ml, and

v) L-Glutamine (Sigma), 200 mM; 5 ml.

2.2.2 Buffers

i) Phosphate buffered saline

The following solutions were autoclaved separately and mixed before use:

8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.2 g KH_2PO_4 in 800 ml double distilled water, (DDW): 0.1 g $CaCl_2$ in 100 ml DDW and 0.1 g $MgCl_2 \cdot 6H_2O$ in 100 ml DDW

ii) Krebs and de Gasquet Buffer (Krebs and de Gasquet, 1964)

The following solutions in DDW were combined: 116 ml of 0.154 M (0.9 %) NaCl; 4 ml of 0.154 M

(1.15 %) KCl; 3 ml of 0.055 M (0.81 %) $CaCl_2 \cdot 2H_2O$; 1 ml of 0.100 M (2.46 %) $MgSO_4 \cdot 7H_2O$; 1 ml of 0.154 M (2.68 %) K_2HPO_4; 6.5 ml of 0.10 M Na_2HPO_4-NaH_2PO_4 buffer solution, pH 7.4 at 25°C. The 0.10 M Na_2HPO_4-NaH_2PO_4 buffer solution was prepared according to the method of Gomori (1955). It had a pH of 7.4 at 25°C: 8.1 ml of 0.10 M (1.42 %) Na_2HPO_4 was mixed with 1.9 ml of 0.10 M (1.56 %) $NaH_2PO_4 \cdot 2H_2O$ or 4.05 ml of 0.20 M (2.84 %) Na_2HPO_4 was mixed with 0.95 ml of 0.20 M (3.12 %) $NaH_2PO_4 \cdot 2H_2O$ and diluted to 10 ml with DDW.

2.2.3 Solutions

All solutions were made in PBS unless otherwise stated.

i) Tris buffered isotonic-ammonium chloride solution (ACT solution)

Nine volumes of 0.83 % aqueous NH_4Cl was mixed with 1 volume of Tris buffer (20.594 g Tris base per liter). Final pH was adjusted to 7.2 with 1 N HCl. Solution was sterilized by filtering through millipore.

ii) Trypan blue solution

A 0.4 % solution of trypan blue (Metheson, Coleman and Bell, Ohio, N.J., U.S.A.) was made in saline (or PBS). This stock solution was diluted to 0.1 % with PBS for use in assaying the viability of lymphocytes.

iii) Tricosanthin (TCS) solution

A stock solution of 2 mg/ml was prepared in PBS, millipore filtered and kept at 2 °C.

iv) Leishman's stain

A 0.15 % of Leishman's stain (Sigma) was prepared in methanol.

v) Mitomycin C solution

Mitomycin C (Sigma) was supplied in vials containing 2 mg powder. A solution of 50 µg/ml was prepared by dissolving 2 mg powder in 40 ml plain RPMI asceptically.

vi) Concanavalin A (Sigma)

A stock solution of 90 µg/ml in PBS was prepared, millipore filtered and kept frozen. The stock was diluted to 30 µg/ml for use in experiments. The final concentration was 3 µg/ml in all experiments unless otherwise stated.

vii) Lipopolysaccharide, LPS (Sigma) (L-5130) from Salmonella typhimurium

A stock solution of 500 µg/ml in PBS was prepared, millipore filtered and kept frozen. The stock was diluted to 25 µg/ml for use in experiments. The final concentration was 2.5 µg/ml in all experiments unless otherwise stated.

viii) Glucose uptake assay reagents

PGO enzymes (Stock No. 510-6)

These were supplied in pre-weighed capsules.

Each capsule contained 500 International units of glucose oxidase (Aspergillus niger), 100 Purpurogallin units of peroxidase (horseradish) and buffer salts. The capsules were stored in the refrigerator at 0-5°C. To prepare the Combined Enzyme-Colour Reagent Solution, the contents of one capsule were added to 100 ml distilled water in an amber bottle which was then inverted several times with gentle shaking to dissolve the contents. The Colour Reagent Solution (1.6 ml) was then added and mixed. The resulting Combined Enzyme-Colour Reagent Solution was stable for up to 1 month at 0-5°C unless turbidity or colour formed. If this happened the mixture was discarded.

O-dianisidine dihydrochloride (Stock No. 510-50)

To prepare the Colour Reagent 50 mg of O-dianisidine dihydrochloride was dissolved in 20 ml water. The reagent was stable for 3 months in the refrigerator at 0-5°C.

Glucose standard solution (100 mg/100ml)

100 mg of glucose was dissolved in distilled water and 2.5 ml perchloric acid was added and the solution diluted to 100 ml with distilled water. The solution was stored at room temperature and discarded if any turbidity

developed. (Commercially available glucose contained ca. 9 % moisture, thus the true concentration of the Standard was 91 mg/100ml). Standards of other concentrations (e.g. 50, 150, 200 mg/100ml etc.) were prepared in a similar way.

Perchloric acid (0.33 M)

2.85 ml of 70 % perchloric acid ($HClO_4$) was diluted to 100 ml with distilled water. This was used for removing proteins from solutions. Excess of $HClO_4$ was not removed from the solutions. Allowance was made in the buffer used in the Enzyme Reagent for neutralizing the $HClO_4$ (Krebs et al., 1963).

ix) Lactic acid production assay reagents

Lactic dehydrogenase (Stock No. 826-6)

This was supplied in 2.5 ml bottles which contained an LDH suspension isolated from beef heart in ammonium sulphate. It was approximately 1000 units per ml when prepared. The suspension was stored at 0-5°C and had to be mixed to make the suspension uniform before use.

Glycine buffer (Stock No. 826-3)

This was supplied in 100 ml bottles containing glycine and hydrazine, pH 9.2. Chloroform was

added as preservative. It was stored at 0-5°C.

NAD (B-nicotinamide adenine dinucleotide, Stock No. N-7004)

This was prepared from yeast, Grade III (approx. 98 % pure) and supplied in 600 mg bulk packages. It was stored desiccated below 0°C.

Lactic acid standard solution (Stock No. 826-10)

This contained L(+)-lactic acid, 0.40 mg/ml with preservative added. It was stored at 0-5°C and diluted 1:5 times (1 ml Standard Solution to 5.0 ml with water) before use. The diluted solution was discarded after 1 day.

Perchloric acid 8 % (w/v)

This was prepared by diluting 7 ml of 70 % (w/v) perchloric acid to 100 ml with water. Cells were prepared and incubated as for the glucose uptake assays. 0.4 ml of ice-cold 8 % $HClO_4$ and 0.1 ml of clear supernatant was used for the assay. Two blank solutions were also prepared which contained $HClO_4$ instead of sample. (Lactic acid in the $HClO_4$ supernatant was stable for at least 1 week at 0-5°C). To each test tube containing the

supernatant or blank, 1.4 ml of Lactic Acid Reagent mixture was added. The reagent was prepared by mixing the following:
100 mg β-NAD; 20 ml glycine buffer; 40 ml distilled water and 1.0 ml LDH suspension.

2.3 Extraction and Characterization of Trichosanthin

2.3.1 Extraction

All procedures were performed at 4°C unless otherwise stated.

Dried Tianhuafen, root tuber of Trichosanthes kirilowii, was obtained from local herb market. The tuber was soaked in distilled water for 2 days, cut into small pieces, homogenized and then filtered through cheese-cloth. The juice was centrifuged at 5000 rpm for 15 minutes to remove insoluble materials. The resulting supernatant was freeze-dried to get crude powder of Tianhuafen for further purification.

The purification process was essentially that of a combination of acetone fractionation, ammonium sulphate precipitation and ion-exchange chromatography on CM-Sepharose CL-6B column, as represented schematically in Fig. 2.1.

i) Acetone fractionation

Crude powder was dissolved in cold water and the pH was adjusted to 4.0 with 2 N HCl. Then 0.5 v/v of cold acetone was slowly added to the

solution. After standing for 1 hour, the supernatant was collected by centrifugation, and cold acetone was again added to 1.5 v/v. After centrifugation, supernatant AP4 was dialysed against distilled water and lyophilized.

ii) Ammonium sulphate precipitation

AP4 was dissolved in cold water. Solid ammonium sulphate (243 g/l) was added slowly to the solution to make 40 % saturation. The solution was stirred further for 30 minutes, then the precipitated protein was removed by centrifuging at 7,000 rpm for 15 minutes. The supernatant S_{40} was dialysed and lyophilized.

iii) Ion-exchange chromatography

S_{40} was dissolved in phosphate buffer (0.05 M, pH 6.4) and applied to a column of CM-Sepharose CL-6B (30 x 1.7 cm), which was equilibrated and eluted with the same buffer. Elution was first carried out with 0.05 M phosphate buffer, pH 6.4, during which 3 peaks were obtained. Subsequent gradient elution with 0-0.3 M PBS, pH 6.4 gave rise to 3 more peaks. The slowest moving peak, which was most distinct and symmetrical, was identified to be Trichosanthin by molecular weight, and amino acid composition analysis.

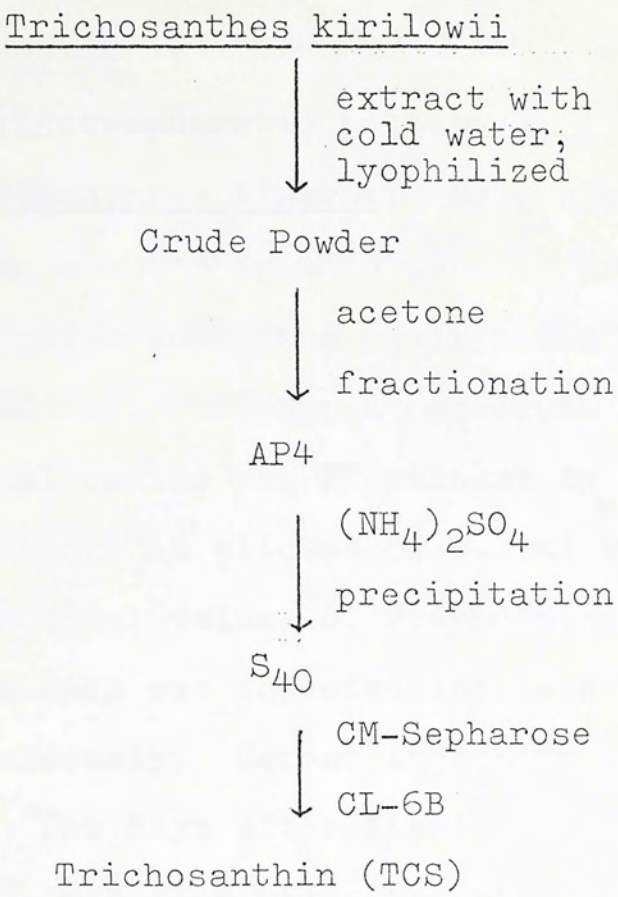

Fig. 2.1 Isolation scheme of Trichosanthin

2.3.2 Characterization

i) Immunoelectrophoretic pattern

preparation of antiserum: Male albino rabbits weighing about 3 Kg were used to produce polyspecific antiserum against the Trichosanthes preparations. Trichosanthes crude powder (15 mg/ml) in normal saline was sterilized by millipore filtration. An aliquot of 0.5 ml was emulsified with an equal volume of Freund's complete adjuvant. The emulsion was injected intramuscularly and subcutaneously. Second injection was given 10 days later. Ten days after the last immunization, booster injection was given with 4 times (60 mg/ml) the initial amount of antigen. During the immunization, the titre of the antiserum was monitored by immunoelectrophoresis and ring test.

immunoelectrophoresis: Immunoelectrophoresis was done according to the method of Grabar and Williams (1953) with slight modification. About 5 ml of a 1 % agarose solution (type II) in barbital buffer (I = 0.025, pH 8.6) was poured onto a clean glass plate (10 x 5 cm) placed on a horizontal surface. When the agarose gel was set, it gave a gel of 1 mm thickness. Three circular wells, 2 mm in diameter, were cut with a gel puncher about 1.6 cm apart. Two rectangular troughs 1-2 mm in width were cut with a surgical

knife, midway between the wells, about 7 mm from each well. Samples under investigation were placed in the wells. Electrophoretic separation was carried out in barbital buffer (I = 0.025, pH 8.6) for 45 minutes at a potential of 50 V/10 cm, measured directly on the plate. Electrical contact between the agarose and the electrode vessels was made with strips of Whatman 3 mm filter paper moistened in the same buffer. After electrophoresis, the rectangular troughs were filled with the antiserum and the gel was incubated overnight in a humid chamber at room temperature. After the development of precipitin arcs, the non-precipitated proteins were removed by washing the gel in 0.9 % NaCl for 3 days. The precipitin arcs were stained with 0.1 % Amido Black 10B and destained in 7 % acetic acid until the background was clear. The gel was then covered with cellophane and allowed to dry at room temperature.

ii) Sodium dodecylsulphate (SDS) gel electrophoretic pattern

electrophoresis: Electrophoresis on polyacrylamide slab gel containing 0.1 % SDS was performed by the method of Laemmli (1970) with slight modifications. A 10 % separating gel (0.375 M Tris, pH 8.8) was made between 2 glass plates (13 x 16 cm) with 3 perspex spacers of thickness 1.5 mm. The glass

plates were clamped together by stationery clips and the three outer edges were sealed with 1 % agar to prevent leakage. After polymerization of the separating gel, a 3 % spacer gel (o.125 M Tris pH 6.8) was added with insertion of a sample well forming comb. The comb was made from 1.5 cm perspex sheet with 13 teeth, each 0.6 cm wide. After polymerization, the comb as well as the lower spacer were removed and the slab was mounted on the electrophoresis apparatus. The sample solution was diluted 1:5 with sample buffer containing 2 % SDS with or without 2 % mercaptoethanol to give a final protein concentration of 1-2 mg per ml. The solution was incubated at 100°C for 2 minutes. Sample solutions of 10-50 µl was applied to each well. Electrophoresis was performed at a constant power of 10 W at room temperature which takes about 75 minutes for the tracking dye to reach the bottom of the gel. At the end of the run, the slab was disassembled. The gels were placed in staining solution of 0.25 % Coomassie Brilliant Blue R250 for 2-4 hours at room temperature. Background stain was removed by destaining overnight in solution containing acetic acid, ethanol and water (1:3:10 v/v) in the Bio-Rad Model 202 Diffusion Destainer.

iii) molecular weight determination by Gel filtration exclusion chromatography: Sephadex G-100 (SF) was washed well with PBS (pH 7.2), poured into a column (2 x 83 cm), and equilibrated extensively with PBS before application of the sample in 1 ml. Chromatography was performed at room temperature at a flow of 8 ml/hour. The column was calibrated with the following standard marker proteins: bovine plasma albumin (67,000), ovalbumin (45,000), α-chymotrypsinogen A (25,000) and cytochrome c (12,400).

iv) Glycoprotein stain

The presence of carbohydrate moieties in TCS was determined by periodic acid-schiff (PAS). Staining procedure according to Glossmann and Neville (1971). TCS and crude powder, after electrophoresis in polyacrylamide slab gel containing 0.1 % SDS, were washed continuously with 3 liters of 40 % methanol and 7 % acetic acid overnight. Gels were oxidized for 1 hour at 4°C with 1 % periodic acid in 7 % acetic acid in the dark. The excess periodic acid was leached out by washing the gels in 7 % acetic acid overnight. Gels were incubated for 1 hour in Schiff's reagent according to Fairbanks (1971) at 4°C in the dark. The gels were stored in Schiff's reagent or in 1 % $Na_2S_2O_5$ in 0.1 N HCl.

v) Amino acid composition analysis

The method of Spackman et al. (1958) was employed for analysing the amino acid composition of TCS. Protein samples (1 mg) were hydrolysed with 1-2 ml of constant boiling HCl (5.7 N) at 110°C for 24 hours in sealed evacuated tubes. After hydrolysis, the acid was removed in a vacuum desiccator over NaOH pellets. The hydrolysate was redissolved in 2 ml of 0.2 M sodium citrate buffer pH 3.25 and suitable aliquots were taken for chromatographic analysis on a Beckman Model 120C amino acid analyser.

vi) Assay of abortifacient activity

Mature female WHT mice weighing 25 to 35 g were caged with fertile males. The presence of copulation plug the next morning was designated day 1 of pregnancy (PD1). Trichosanthin preparation of 0.1 mg/25 g body weight in normal saline were administered intraperitoneally on day 12 of pregnancy (PD12). Mice were autopsied on PD14. The total number of uterine implantation sites were recorded and the sizes were measured. The uterine implantation sites were qualitatively defined as either live fetuses, dead or resorbing fetuses.

2.4 In vitro Studies

2.4.1 Lymphocyte culture

i) Preparation of splenocyte single cell suspension.

CBA mice were killed with ether anaethetization. Spleens were removed asceptically and then meshed on stainless steel meshes. Cell clumps were removed by a quick spin. Red blood cells were lysed by ACT solution. Cell concentration was determined by counting in a hemocytometer. The cell concentration was adjusted to 5×10^6/ml in complete medium in all experiments unless otherwise stated.

ii) Culture of cells to investigate biosynthetic activities.

Cells were cultured in Linbro flat-bottomed 96-wells microtiter plates inside an incubator maintained at 36°C, supplied with 10 % CO_2 in air. Unless otherwise stated, culture period was 48 hours, after which each well was pulsed with 0.5 µCi of one of the labeled precursors: (Methyl - ^{3}H) Thymidine, 25 Ci/mmol (Amersham); (5-^{3}H) Uridine, 25 Ci/mmol (New England Nuclear); L-(4,5-^{3}H) Leucine, 1 Ci/mmol (Amersham) for 6 hours. Radioactivity incorporated was harvested by a Titertek multiple sample cell harvester on glass fibre filter (Whatman GF/C). The GFC was suspended in scintillant (0.5 % PPO, 2,5-diphenyloxazole, in toluene; 7 ml) and counted in LS-3000 Beckman Liquid Scintillation Counter with an efficiency for ^{3}H-isotopes of 60%.

2.4.2 Viability and agglutination

Cells were mixed with an adequate volume of trypan blue solution, and then counted in a hemocytometer. Dead cells were stained blue while viable cells were colourless and refractile. Agglutination was observed under microscope daily during the culture period.

2.4.3 Mixed lymphocyte reaction

Single cell lymphocyte suspensions of CBA and WHT mice were prepared as stated. CBA cells were adjusted to 5×10^6 per ml and WHT cells were mitomycin C treated.

i) Mitomycin C treatment: WHT cells were adjusted to 4×10^6/ml. One volume of this cell suspension was mixed with one volume of mitomycin C (50 µg/ml). The final concentration of mitomycin C was 25 µg per 2×10^6 cells. The mixture was incubated at 37°C in an atmosphere of 5 % CO_2 in air for 30 minutes. Cells were then washed thrice with plain medium and resuspended to 5×10^6/ml.

ii) Culture

Eighty µl each of WHT and CBA cells were added to each well in Linbro flat-bottomed microtitre plates. In control wells, 80 µl of one cell type only was added. In studies of response kinetics of the reaction, cells were cultured for 48, 72 and 96 hours, and then pulsed with 0.5 µCi of either ^{3}H - TdR, ^{3}H - UdR or ^{3}H - Leu, and harvested 20 hours later. Effects of TCS on this MLR was investigated by addition to each well 20 µl of different concentrations of TCS. Wells to study ^{3}H - TdR uptake were labeled at 72 hours and harvested 20 hours later. Wells to study ^{3}H - UdR and ^{3}H - Leu uptake were labeled at 48 hours and harvested 20 hours later. The radioactivity incorporated was counted as described.

2.4.4 Glycolytic activities

i) Cell culture

Spleen cells isolated from 12 CBA mice were pooled and prepared at 1 x 10^{7} cells /ml. Thirty ml of the cell suspension was delivered into each of 4 T - 75 culture flasks:

flask 1: 30 ml cell + 4.5 ml PBS

flask 2: 30 ml cell + 1.5 ml PBS + 3.0 ml con A

flask 3: 30 ml cell + 1.5 ml TCS + 3.0 ml PBS

flask 4: 30 ml cell + 1.5 ml TCS + 3.0 ml con A

Con A of 30 μg/ml was used, final concentration 3 μg/ml.

TCS of 2000 μg/ml was used, final concentration 100 μg/ml.

The flasks were incubated at 37°C under 10 % CO_2 in air for 48 hours.

The incubated cells were then washed twice with glucose-free Krebs and de Gasquet buffer and incubated in the same buffer for 1 hour to exhaust endogenous glucose. Then the cells were resuspended in this buffer with 5.56 mM glucose at a cell concentration of 3.5×10^7 viable cells/ml. Two point six ml of this cell suspension was transferred to tissue culture tubes and incubated for another 2 hours.

At time zero, 1 and 2 hours after incubation, duplicates of 0.2 ml cell suspension from each tube was taken out for glucose and lactate assays.

ii) Glucose uptake

The method used for determining glucose uptake was described by Huggett and Nixon (1957) and Jakobsen (1960). The procedure was that of the

Sigma Technical Bulletin No.510 described by Bergmeyer and Bernt (1974). Sigma reagents were used. Briefly, protein was precipitated by mixing 0.2 ml suspension with 2 ml $HClO_4$ (0.33 M) and then centrifugation. Two ml of the Combine-Enzyme Colour Reagent was added to 0.2 ml supernatant (duplicate). The mixture was allowed to react at room temperature for 45 minutes. Absorbance at 450 nm was then read.

iii) Lactic acid determination

This method was described by Peleiderer and Dose (1955) and Hohorst (1957). The present procedure was conducted according to Sigma Technical Bulletin No. 826-uv and the procedure described by Gutmann and Wahlefeld (1974). Briefly, 0.2 ml cell suspension was deproteinized by mixing with 0.4 ml $HClO_4$ (8 %) and then centrifuging. Duplicates of 0.1 ml supernatant were allowed to react with 1.4 ml Lactate Reagent at room temperature for 45 minutes. Absorbance was then read at 340 nm.

2.4.5 Rate of oxygen consumption

Single cell suspension was prepared at 1 x 10^7/ml. Con A used was 90 µg/ml and TCS, 2000µg/ml.

i) Oxygen consumption rate of con A stimulated splenocytes as a course of time

Two groups of culture (4 sets) were conducted:

group 1 (cell control): 3.5 ml cell + 0.5 ml PBS

group 2 (stimulated): 3.5 ml cell + 0.2 ml PBS
+ 0.3 ml con A

The cultures were incubated at 37°C, 10 % CO_2 in air. At 0, 16, 39 and 43 hours, the oxygen level in the culture was monitored polarographically by an oxygen monitor, YSI Model 53. Group one culture was transferred to the sample chamber for recording the rate of oxygen consumption for 20 minutes. Then group 2 was also recorded. The oxygen consumption rate was calculated from the printout chart. The rate was expressed as the percentage decrease of oxygen saturation of the culture with time, %/minute.

ii) Effect of TCS on oxygen consumption rate

Four groups (2 sets) of culture were conducted:

group 1 (cell control): 3.5 ml cell + 0.5 ml PBS

group 2 (con A control): 3.5 ml cell + 0.3 ml con A + 0.2 ml PBS

group 3 (TCS control): 3.5 ml cell + 0.2 ml TCS + 0.3 ml PBS

group 4 (experimental): 3.5 ml cell + 0.3 ml con A + 0.2 ml TCS

After 8 and 12 hours of culture, the oxygen consumption rate of these cultures were recorded consecutively for 20 minutes.

2.5 In vivo Studies

2.5.1 Delayed hypersensitivity reaction

The response was tested in two strains of inbred mice, CBA and WHT. Mice were divided into three groups: experimental (E), control 1 (C1), and control 2 (C2). Each group consisted of 5 animals. Experimental animals were injected with TCS before sensitization and challenge. Control 1 was injected with PBS, sensitized and challenged whereas control 2 was challenged only.

On day 0, experimental animals were injected intraperitoneally with TCS at a dose of 0.2 mg/25 g body weight in 0.2 ml of PBS. Control 1 was injected with 0.2 ml of PBS, the carrier. On day 2, the mice were sensitized intravenously by injecting 1×10^6 sheep red blood cells in 25 µl into the tail vein. On day 6, the normal footpad thickness was measured by using a Schnell taster caliper. The right foot pad was then challenged subcutaneously by injection of 1×10^8 sheep red blood cells in 25 µl to the right foot pad. The left foot pad (unchallenged control) was injected with 25 µl of PBS. The footpad thickness was measured 24, 48 and 72 hours after challenge.

2.5.2 In vivo TCS treatment and subquent in vitro mitogenic stimulation

CBA mice were ether anaesthetized and then injected intraperitoneally with 0.2 mg of TCS in 0.2 ml PBS per 25 gm body weight. Controls received 0.2 ml PBS. Mice were treated twice, once on day zero, once on day two and was sacrificed on day four. Spleens from these mice were isolated, and single cell suspensions prepared separately. Their responses to mitogenic stimulation by con A and LPS were tested as stated in Section 2.4.1 above.

2.6 Mechanistic Studies

2.6.1 Binding of ^{125}I-con A

i) Iodination

Con A was radiolabeled with carrier free Na^{125} I (Amersham, 16.15 mCi / µg of iodine) by using chloramine T. A 2 mg/ml con A solution was prepared in PBS. The reaction was carried out in a borosilicate disposable test-tube. Ten microliter of Na^{125} I (16.15 mCi / µg) was added to the con A solution. Then was added 50 µl of chloramine T (BDH, 4 mg/ml in PBS) and allowed to react for exactly one minute. Then 0.2 ml sodium metabisulphite (BDH, 2.4 mg/ml in PBS) was immediately

added to stop the reaction. The whole mixture was then charged onto a Sephadex G-25 column (1.6 cm x 35 cm). Eluates were collected in 2 ml aliquots at 15 drops per minute. The migration of radioactivity was monitored by a Geiger Counter. Two peaks of radioactivity were detected. The faster moving one was con A and the slower, unreacted ^{125}I. The specific activities of the eluates were determined by counting an aliquot of the eluate in a gamma counter (Beckman Gamma 4000) and then determining the protein content (Lowry et al. 1951).

ii) The binding assay

In each assay, 0.8 x 10^6 CBA splenocytes were used. The cells were incubated in the presence or absence of varying concentrations of TCS together with a fixed amount of ^{125}I con A ($\sim$ 25,000 cpm) in a total volume of 0.2 ml of complete medium of RPMI 1640. Reactions were allowed to proceed to equilibrium at 37°C in an atmosphere of 10 % CO_2 in air for 1 hour. Cells were then washed thrice with plain RPMI and the cell bound radioactivity determined by a Beckman Gamma 4000 Counter.

2.6.2 Pretreatment experiments

Splenocytes from CBA mice were prepared, cultured, labeled, harvested and incorporated radioactivity determined under the same conditions as stated in 2.4.1. However, some manipulations, described below, were performed to observe the effects of various pretreatments on subsequent lymphocyte blastogenesis.

i) Con A pretreatment

At the start of culture, 160 µl cell and 20 µl con A were added to microtitre plate. Trichosanthin (1000 µg/ml, final 100 µg/ml) was added and mixed at 0, 2, 4, 8, 12, 24 hours. The culture was labeled at 48 hours and harvested 6 hours later.

ii) Trichosanthin pretreatment

At the start of culture, 160 µl cell was added to each well. Trichosanthin (1000 µg/ml) was added and mixed at 0, 2, 4, 6 and 12 hours. Con A was added at 24 hours. Culture was incubated for 48 hours more, then labeled and harvested.

iii) Con A and Trichosanthin pretreatment

Eight volumes of cell suspension was cultured with 1 volume of con A and 1 volume of TCS in a T-25 culture flask. At 0, 4, 6, 8, 12, 24, 32 and 48 hours, 2 ml aliquots were taken out

from the flask and washed thrice with 0.15 M α-MM in PBS. Alpha-MM was used to remove any con A tightly bound to cell surface. The cells were resuspended to 5 million/ml and then recultured with either con A or PBS. Culture was labeled at 48 hours for 6 hours and then harvested.

2.6.3 Blast cell count

Cell smears were prepared on a microscopic slide from 0.2 ml of a cell suspension (0.5×10^6/ml) by a cytocentrifuge (Shandon Southern). The centrifugal speed was 1,500 rpm and the sample was spun for 5 minutes. The circular smear was air-dried and 1 ml of Leishman stain was layered on the slide for 3 minutes. The stain was diluted with 2.5 ml of tap water and washed off with distilled water 10 minutes later. The slide was examined under microscope and different types of cells counted.

CHAPTER III : RESULT AND DISCUSSION

3.1 The Immunosuppressive Protein from Tianhuafen

3.1.1 Isolation

The immunosuppressive protein was isolated from the Chinese drug, Tianhuafen (*Trichosanthes kirilowii*) according to procedures previously developed in our laboratory (Yeung, 1980c; Yeung and Li, unpublished result). In sum, Tianhuafen was extracted with cold water to give a crude powder, which was then purified by a combination of acetone fractionation and ammonium sulfate precipitation. The final purification step was ion-exchange chromatography on CM-Sepharose CL-6B column. The elution profile was shown in Figure 3.1 in which 3 peaks were first eluted by phosphate buffer (0.05M, pH 6.4), subsequent linear gradient elution with 0-0.3 M NaCl in the same buffer gave rise to 3 more peaks. The slowest moving peak, here arbitrarily referred to as protein six (P6), has been found to be active in inhibiting con A-induced lymphocyte transformation in mice.

3.1.2 Purity

The purity of P6 was examined by both immunoelectrophoresis and SDS-gel electrophoresis. Shown in Figure 3.2 is the immunoelectrophoretic pattern of the crude powder and P6. The crude powder was a complex mixture containing more than 4 components as indicated by the number of precipitation arcs. In contrast, P6 gave a single arc and

Figure 3.1 Chromatography of S40 on CM-Sepharose CL-6B column (1.7 x 30 cm). Fractions of 3 ml were collected at a flow 20 ml/hour.

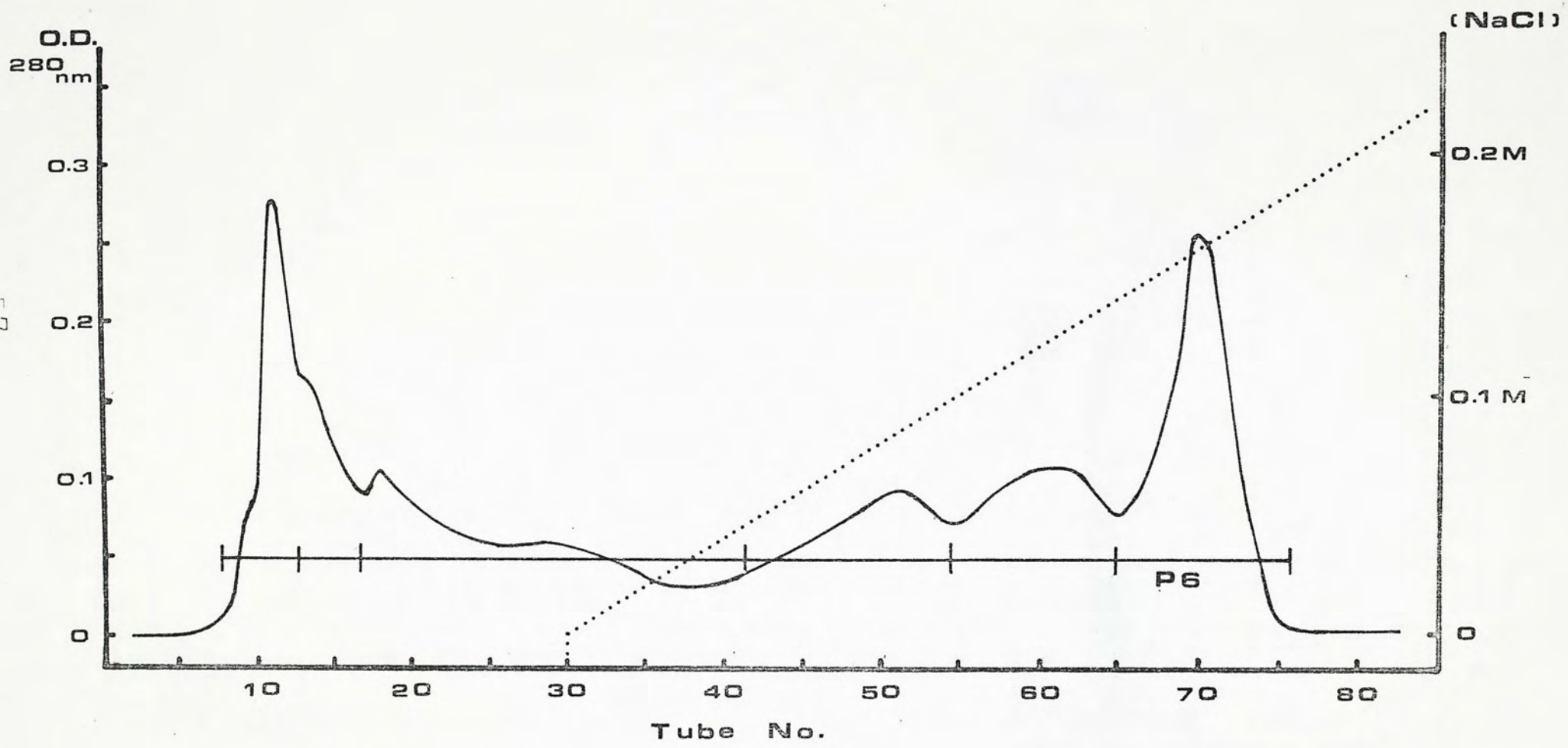

Figure 3.1

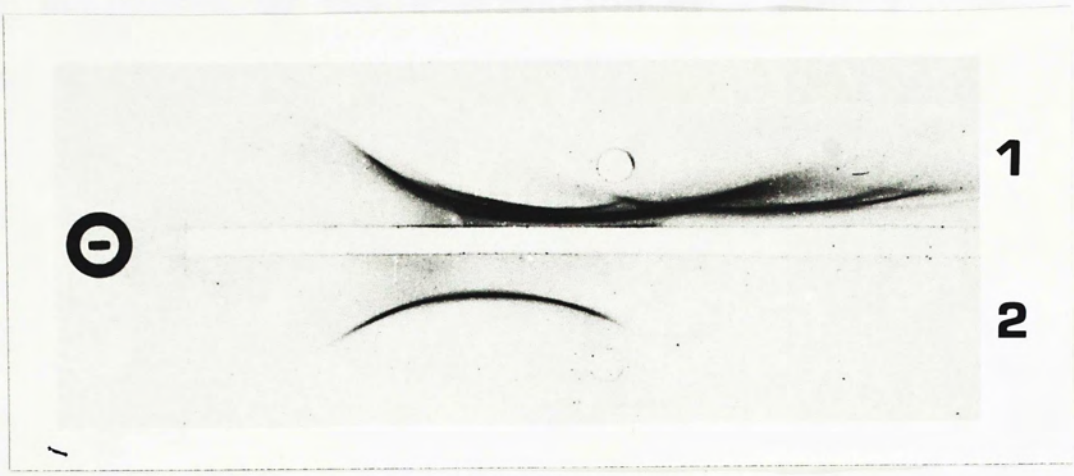

Figure 3.2 Electrophoretic pattern of crude powder (1) and protein 6 (2).

is, therefore, immunochemically pure. The homogeneity of P6 was confirmed by SDS gel electrophoresis. The SDS-gel electrophoretic pattern was shown in Figure 3.3 which showed a single band for P6 but the presence of more than four components in the crude extract. The homogeneity of P6 was further supported by the observation of a single symmetrical peak on gel filtration chromatography on Sephadex G100 (SF) (see Figure 3.4 below) and the detection of a single amino acid, Asp, as the N-terminal residue of P6 (Yeung, unpublished result).

3.1.3 Identity

The Chinese scientists (Wang et al., 1976b) first reported the purification of the abortifacient protein, Trichosanthin (TCS), from Trichosanthes kirilowii by repeated chromatography on CM-Sephadex C-50. They found that TCS was a glycoprotein of molecular weight 18,000. Later studies by Wang and coworkers of the Institute of Organic Chemistry, Shanghai showed that TCS contained very little carbohydrate, if any, and the molecular weight was revised to 24,000 (Wang et al., 1979). The amino acid composition (see Table 3.1) and the N-terminal residue was found to be Asp by the Shanghai group, who is currently working on the primary structure of TCS (Wang, private communication). The purified TCS effectively induced abortion of mid-term pregnant mice at a dose of 0.2 mg/mouse (Wang et al., 1976b).

In the present study, the immunosuppressive protein,

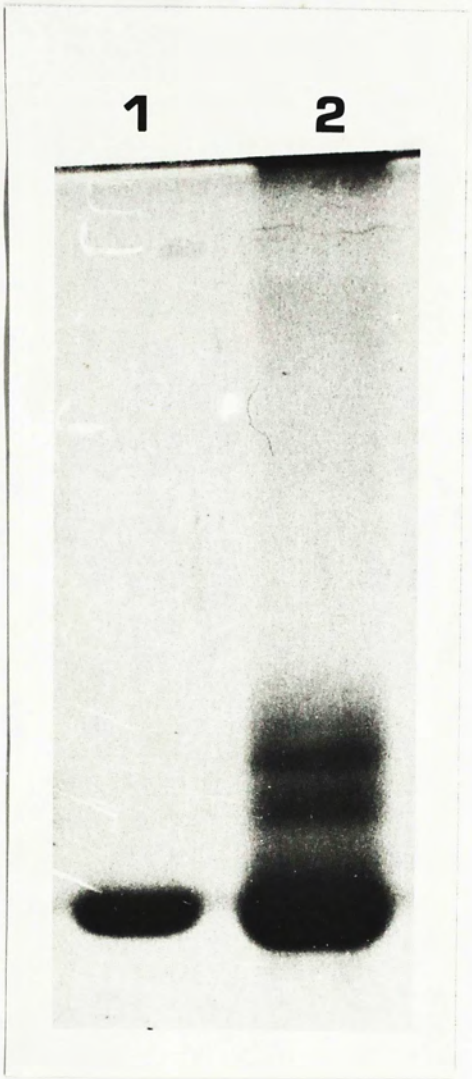

Figure 3.3 SDS gel electrophoretic pattern of crude powder (2) and protein 6 (1).

P6, has been isolated from the same source as TCS by similar procedure. It is, therefore, of much interest to determine whether P6 is identical to TCS. To this end, we first tested the abortifacient activity of P6 in 12-day pregnant mice. The results presented in Table 3.1 show that a single intraperitoneal (i.p.) injection of P6 (0.1 mg/25 g body weight) on day 12 induced abortion in all 7 pregnant mice within 48 hours. The molecular weight of P6 was estimated to be 24,000 by gel filtration. Figure 3.4 shows the elution of P6 as a single, symmetrical peak on Sephadex G-100 (SF) column and Figure 3.5 was the calibration curve obtained by gel filtration with protein markers of known molecular weight. The amino acid composition of P6 was analyzed and the results were presented together with that of TCS in Table 3.2. Almost identical amino acid compositions were observed for P6 and TCS. It is worthwhile to note that Cys is absent and the number of His (1) and Met (3) residues are identical in both proteins. The abundance of hydrophobic amino acids such as Ala, Leu, Ile, Val and Phe is also obvious in both proteins. Furthermore, both proteins were shown to have Asp as the N-terminal amino acid residues. Protein six (P6) is unlikely to be a glycoprotein as no carbohydrate could be detected by PAS stain in gels. Similar result was recently obtained for TCS as mentioned. Based on the above biological and chemical properties, it is concluded that P6 is identical to TCS.

Table 3.1 Midterm abortifacient activity of Protein 6. The day when vaginal plug was observed was taken as PD 1. Mice were treated i.p. with P6 on PD 12 and autopsied on PD 14.

Treatment	0.1 mg P6 per 25 gm body weight	0.2 ml normal saline
No. of mice	7	5
No. of dead fetus / No. of implant. sites	$\frac{10}{10}, \frac{9}{9}, \frac{11}{11}, \frac{14}{14}, \frac{10}{10}, \frac{5}{5}, \frac{8}{8}$	$\frac{0}{12}, \frac{0}{7}, \frac{0}{9}, \frac{0}{12}, \frac{0}{10}$
Total no. of dead fetus / Total no. of implant. sites	$\frac{67}{67}$	$\frac{0}{50}$
No. of aborted mice	7	0
% aborted	100	0

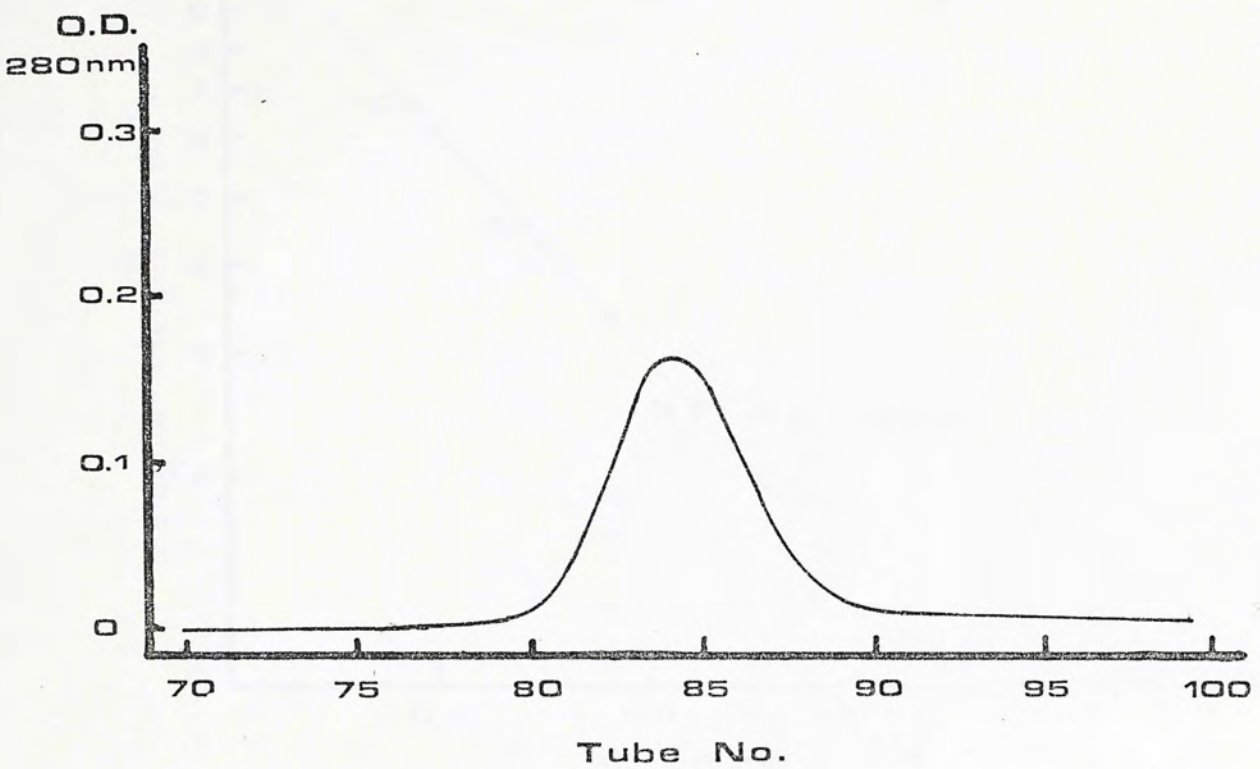

Figure 3.4 Gel filtration chromatographic pattern of P6 on Sephadex G-100 (SF).
Column: 2.0 x 83 cm
Eluent: phosphate buffered saline
Flow rate: 8 ml/hour

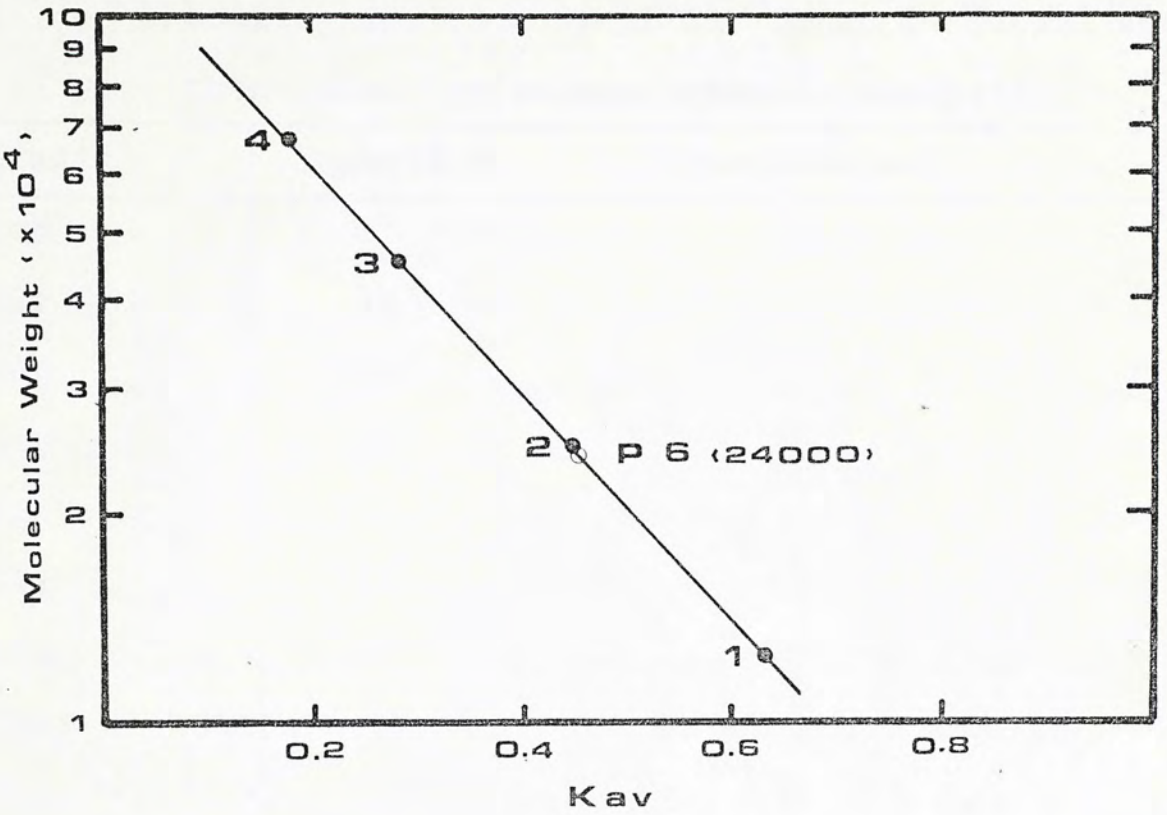

Figure 3.5 Estimation of molecular weight of Protein 6 by gel filtration chromatography on Sephadex G-100 (SF).

M.W. markers used were: 1. cytochrome c(12400) 2. α-chymotrypsinogen A (25000) 3. ovalbumin (45000) 4. bovine plasma albumin (67000)

The M.W. of P6 (O) was 24000

Table 3.2 Amino acid composition of Protein 6 and Trichosanthin.* Protein 6 was hydrolysed by boiling HCl (5.7 N) at 110°C for 24 hours, and then taken for chromatographic analysis.

Amino acid	Protein 6	Trichosanthin
Asp	26	23
Thr	14	15
Ser	22	18
Glu	19	20
Pro	8	6
Gly	11	12
Ala	25	27
Cys	0	0
Val	12	11
Met	3	3
Ile	15	16
Leu	23	25
Tyr	10	11
Phe	8	9
Lys	9	8
His	1	1
Arg	11	11
Total number of residues	217	217

* Results obtained by Professor Y. Wang (汪猷) of the Institute of Organic Chemistry, Shanghai, China.

3.2 Effects of Trichosanthin on Immune Responses of the Mouse In Vitro Studies

3.2.1 Basal synthetic activities of lymphocytes

The dose-dependent effect of Trichosanthin (TCS) on basal syntheses of RNA, protein and DNA of CBA splenocytes was studied. As shown in Figure 3.6 , there was a dose-dependent suppressive effect of TCS on the basal synthetic activities of the splenic lymphocytes. Protein and RNA syntheses seemed to be less sensitive to the inhibitory effect of TCS, reverting to normal levels at 1 μg/ml. DNA synthesis appeared to be more sensitive, significant suppression was observed even at 0.1 μg/ml. Similar differences in the extent of suppression was also observed at higher doses. This may be due to the fact that RNA and protein syntheses precede DNA synthesis (Ling and Kay, 1975). By the time of DNA synthesis, a greater inhibitory effect may have been accumulated.

The viability of cells after 48 hours of culture in the presence of TCS at 100 μg/ml was 79 %, which was the same as the control value. Hence the suppression observed was unlikely to be due to the cytotoxic effect of TCS on splenic lymphocytes.

3.2.2 Mitogenic activation of lymphocytes

i) T lymphocytes

Con A selectively activates T-cells. Andersson et al. (1972b) observed a very narrow dose response

Figure 3.6 Effects of TCS on basal macromolecular syntheses by splenocytes. Splenocytes were cultured with various concentrations of TCS for 48 hours, then labeled with ^{3}H-TdR (●), ^{3}H-UdR (▲) and ^{3}H-Leu (○) and harvested 6 hours later. Each point is the mean ± S.D. of quadruplicate samples.

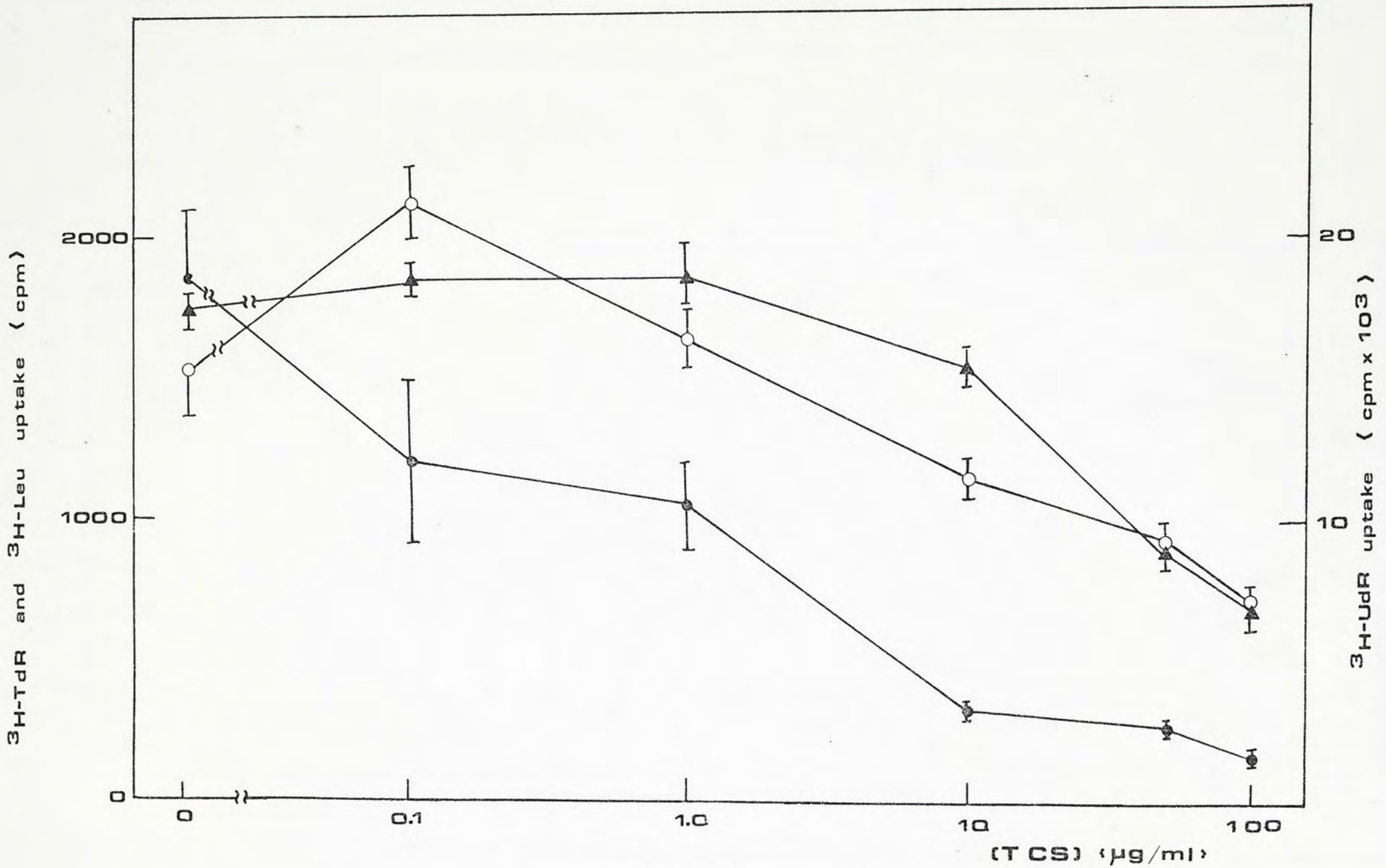
3H-TdR and 3H-Leu uptake (cpm)
2000
1000
0
3H-UdR uptake (cpm x 103)
20
10
0
0.1
1.0
10
100
(TCS) (μg/ml)

Fig 3.6

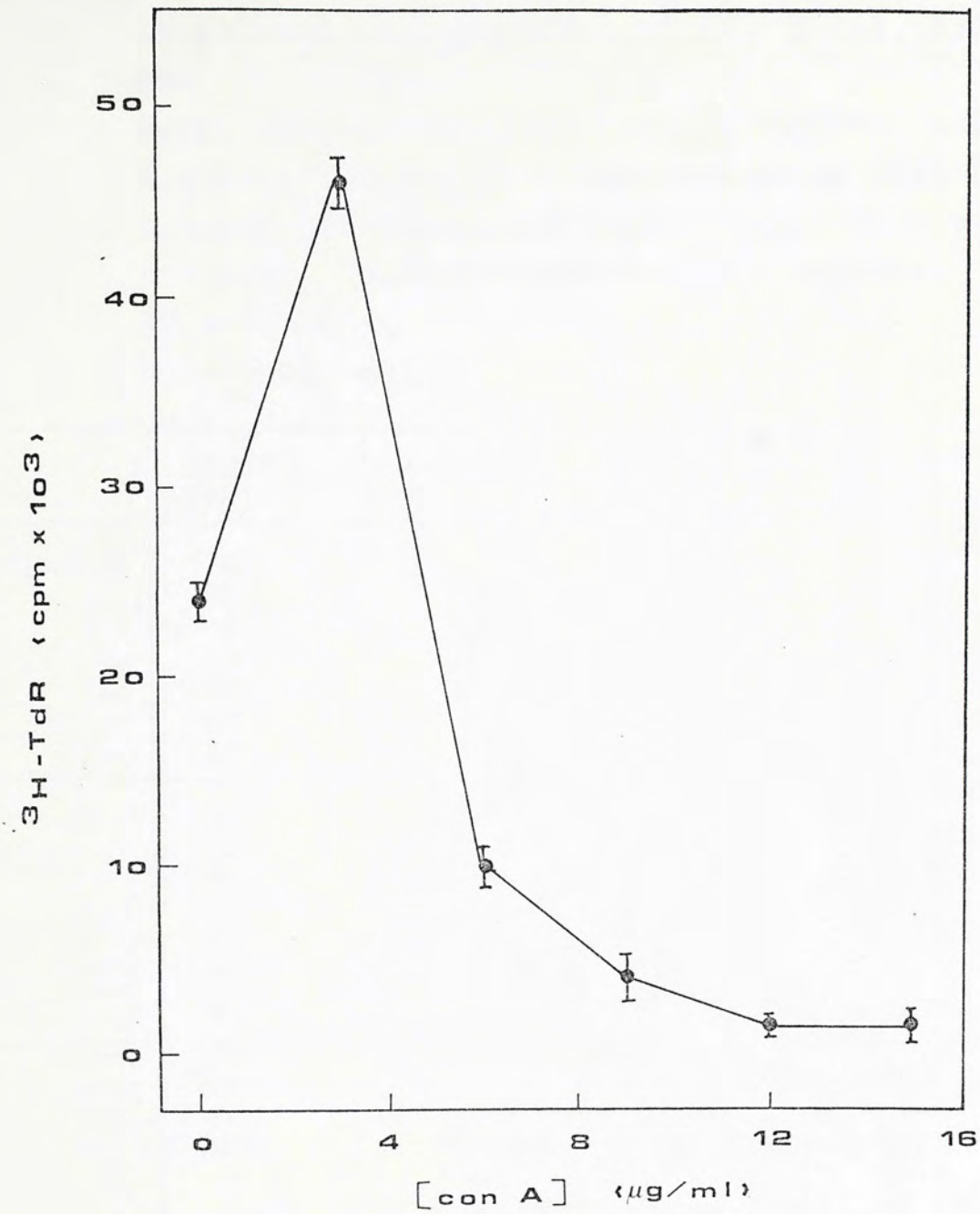

Figure 3.7 Dose-response curve of mouse splenocytes to con A. Splenocytes were cultured with various concentrations of con A for 48 hours, then labeled and harvested. Each point is the mean ± S.D. of quadruplicate samples.

Table 3.3 Dose-dependent inhibition of TCS on con A stimulated incorporation of a) ^{3}H-TdR, b) ^{3}H-UdR and c) ^{3}H-Leu.

Cells were cultured with con A (3µg/ml) and various doses of TCS for 48 h before pulsing with the labeled precursors and harvesting. Each value is the mean ± S.D. of quadruplicate samples.

AC = con A

CC = cell control

	TCS µg/ml	mean cpm ± S.D.	percent inhibition
a) ^{3}H-TdR	100	2508 ± 158	96.5
	50	4058 ± 517	88.3
	10	10381 ± 2817	54.8
	1	16562 ± 1360	22.1
	0.1	21779 ± 1644	0
	CC	1854 ± 249	-
	AC	20731 ± 1615	0
b) ^{3}H-UdR	100	13984 ± 908	100
	50	20633 ± 1273	83.1
	10	23893 ± 1856	65.3
	1	35254 ± 1505	3.1
	0.1	36103 ± 2913	0
	CC	17549 ± 1617	-
	AC	35808 ± 2818	0
c) ^{3}H-Leu	100	1093 ± 78	100
	50	1997 ± 203	95.8
	10	4949 ± 331	68.9
	1	10235 ± 940	20.8
	0.1	13328 ± 1186	0
	CC	1532 ± 293	-
	AC	12521 ± 1087	0

profile of pure T cells to con A, 5 μg/ml being optimal and 1 μg or 10 μg/ml giving a small or no response.

Figure 3.7 shows the dose response of mouse splenocytes to con A. A rather sharp profile peaking around 3 μg/ml was obtained. At concentrations higher than 6 μg/ml, ^{3}H-TdR incorporation was close to the background value. There was a 20 fold stimulation over control at the dose of 3 μg/ml. Unless stated otherwise, this optimal concentration of con A was used in subsequent experiments.

The inhibitory effects of TCS on lymphocyte transformation induced by con A were investigated. Lymphocytes isolated from spleen, lymph nodes and thymus have been tested.

Table 3.3 shows the dose-dependent inhibitory effect of TCS on RNA, protein and DNA syntheses by murine splenic lymphocytes. Complete inhibition was obtained, in all cases, at 100 μg/ml. The same dose-dependent inhibition of con A-mediated blastogenesis in cells from lymph nodes and thymus was also observed (fig. 3.8). TCS did not agglutinate lymphocytes, nor did it affect the con A-induced leucoagglutination.

Rosentreich and Wilton (1975) demonstrated the macrophage-dependency of T-lymphocyte activation

Table 3.3 Dose-dependent inhibition of TCS on con A stimulated incorporation of a) ^{3}H-TdR, b) ^{3}H-UdR and c) ^{3}H-Leu.
Cells were cultured with con A (3μg/ml) and various doses of TCS for 48 h before pulsing with the labeled precursors and harvesting. Each value is the mean ± S.D. of quadruplicate samples.
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	TCS μg/ml	mean cpm ± S.D.	percent inhibition
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	0.1	21779 ± 1644	0
	CC	1854 ± 249	-
	AC	20731 ± 1615	0
b) ^{3}H-UdR	100	13984 ± 908	100
	50	20633 ± 1273	83.1
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	1	35254 ± 1505	3.1
	0.1	36103 ± 2913	0
	CC	17549 ± 1617	-
	AC	35808 ± 2818	0
c) ^{3}H-Leu	100	1093 ± 78	100
	50	1997 ± 203	95.8
	10	4949 ± 331	68.9
	1	10235 ± 940	20.8
	0.1	13328 ± 1186	0
	CC	1532 ± 293	-
	AC	12521 ± 1087	0

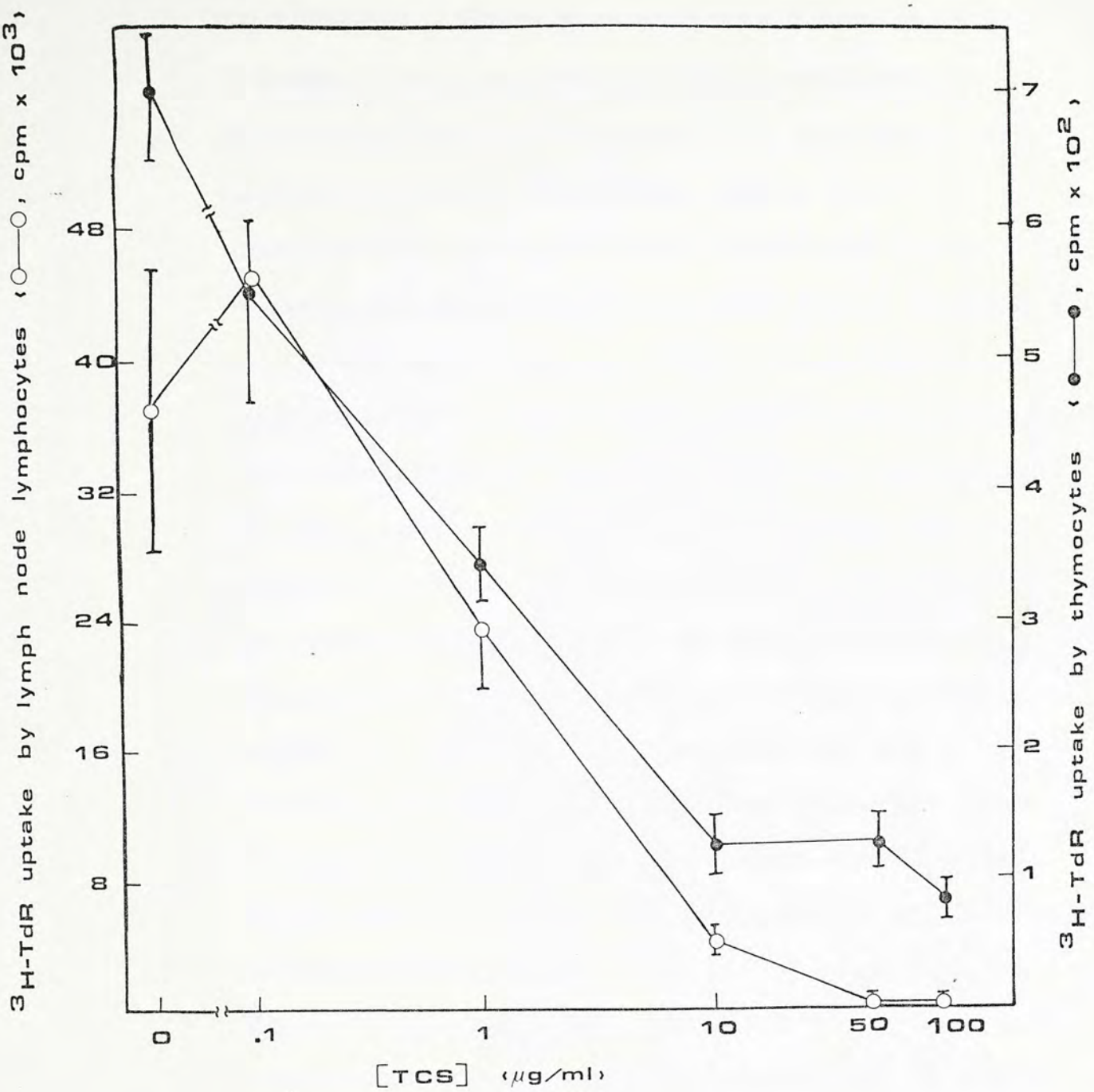


Figure 3.8 Inhibition of TCS on con A-stimulated (^{3}H)--TdR incorporation in CBA thymocytes (●——●) and lymph node lymphocytes (○——○). Cells were isolated, cultured with various doses of TCS in the presence of con A (3 μg/ml) for 48 hours. Cultures were then pulsed with ^{3}H-TdR for 6 hours, and harvested.

by mitogens. Using two adherent nylon wool columns, they prepared a highly purified, macrophage depleted T-lymphocyte population of guinea pig lymph node cells, which were unresponsive to stimulation by PHA and con A. The stimulation was restored by addition of macrophages to the culture. The secretion by macrophages of a lymphocyte activating factor (LAF) which restored the stimulation of pure T-lymphocytes by mitogens was also demonstrated. Since the thymus consists essentially of T cells (Barrett, 1978), thymocytes are seldom used for study of the proliferative response to mitogens as they usually give poor responses. This probably explain why the mitogenic response of thymocytes is so low (679 cpm) when compared to that of spleen cells (20731 cpm) or lymph node cells (36988 cpm).

ii) B lymphocytes

Peavy et al. (1973) reported the mitogenicity of endotoxins from Salmonella typhimurium on mouse spleen cells. The stimulating effect is selectively for mouse B cells. LPS (endotoxin) is also a B cell mitogen in the guinea pig and the rat, although the mouse, for unknown reasons, is much more sensitive to the mitogenic action of LPS than any other species so far studied. It has little mitogenic activity for human, rabbit

or monkey lymphocytes. Andersson *et al.* (1972a) found that LPS would cause an increase in the total immunoglobulin synthesis in stimulated lymphocytes. They used an optimally stimulating dose of 10 μg/ml.

In the present study, LPS from *Salmonella typhimurium* was used. A dose-response experiment was performed on mouse splenocytes to determine the optimally stimulating concentration (Figure 3.9). It was observed that mouse splenocytes were stimulated by LPS at a wide range of concentrations: 50 μg/ml to 0.625 μg/ml. Considerable stimulation was obtained at all doses tested. At 2.5 μg/ml, the greatest amount of ^{3}H-TdR was incorporated. Hence, this concentration was chosen for further experiments.

Figure 3.10 shows the dose-dependent inhibition of TCS on LPS-mediated splenocyte transformation. Complete inhibition was obtained at 50 μg/ml. TCS at a concentration of 1 μg/ml or less had no significant inhibitory effect. Lipopolysaccharide itself is only slightly agglutinating on which TCS did not show any inhibition.

3.2.3 Mixed lymphocyte reaction

The effect of TCS on one-way MLR of CBA and WHT mouse splenocytes was investigated. Unidirectional MLR can be

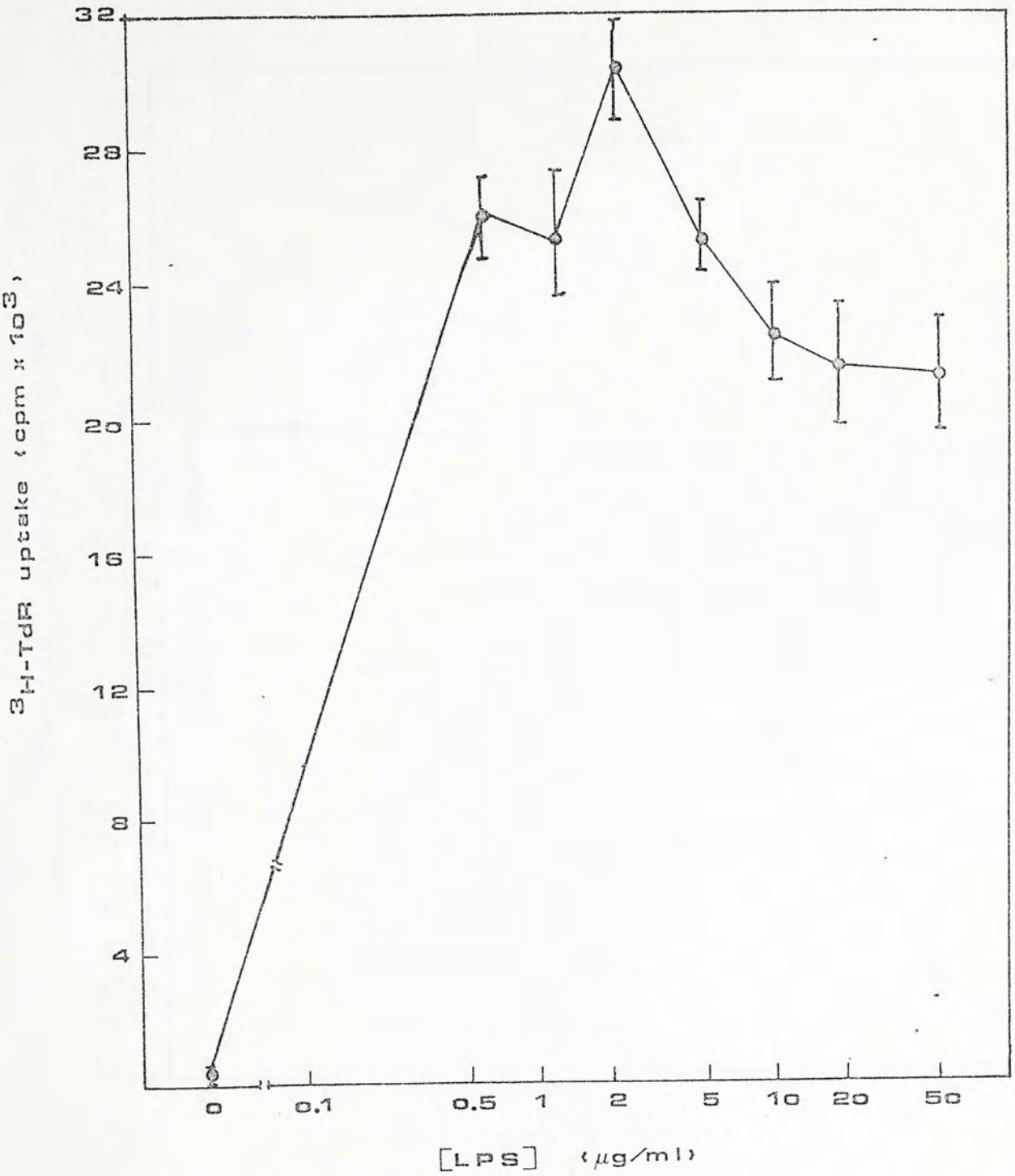


Figure 3.9 Dose-response curve of mouse splenocytes to LPS. Splenocytes were cultured with various concentrations of LPS for 48 hours, then labeled with ^{3}H-TdR and harvested 6 hours later. Each point is the mean ± S.D. of quadruplicate samples.

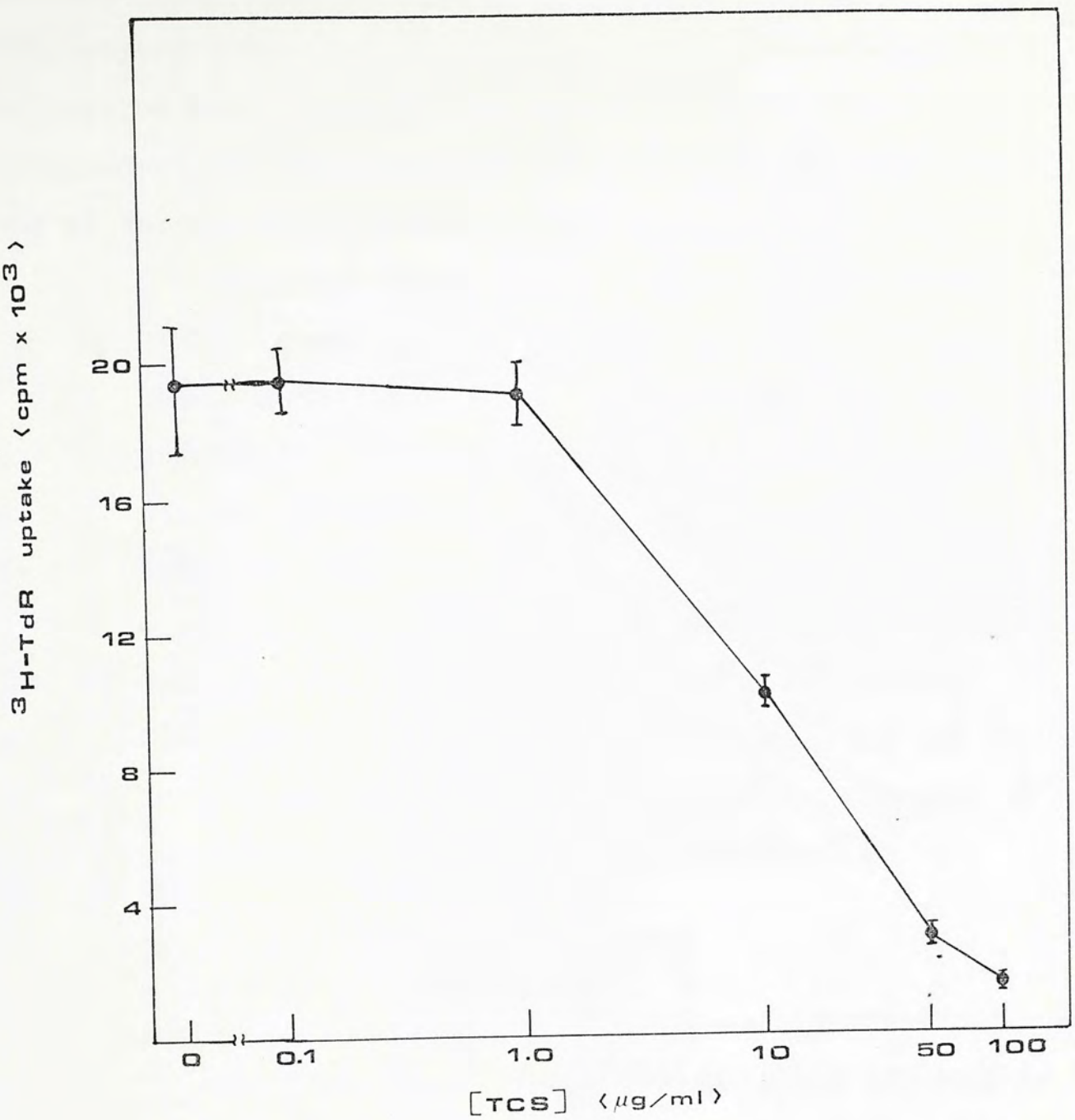


Figure 3.10 Inhibition of TCS on LPS-stimulated (^{3}H)-TdR incorporation by mouse splenocytes. Splenocytes were cultured with various concentrations of TCS in the presence of 2.5 μg/ml LPS for 48 hours, then labeled with ^{3}H-TdR and harvested 6 hours later. Each point is the mean ± S.D. of quadruplicate samples.

achieved by 2 approaches: genetically by the use of lymphocytes from a heterozygote F_1 animal (stimulator) cultivated with cells from an inbred parental strain (responder), or biochemically by inhibiting DNA synthesis in one of the two cell populations with X-irradiation or mitomycin C treatment (Meo, 1979). In the present study, one-way MLR was performed as described by Bach and Voynow (1966), using mitomycin C to inhibit DNA synthesis in one of the 2 populations. Preliminary experiments showed that a combination of untreated CBA cells (C) and mitomycin C treated WHT cells (Wm) i.e. CWm gave a greater MLR than vice versa, i.e. WCm. Hence the combination of CWm with a cell number ratio of 1 to 1 was used for subsequent studies. Figure 3.11 depicts the stimulation indexes of the MLR as monitored by ^{3}H-UdR, ^{3}H-Leu and ^{3}H-TdR uptake as a course of time. The stimulatory index, S_I, was calculated as

$$\frac{\text{Cpm of CWm}}{\text{Cpm of C + Cpm of Wm}}$$

A value of S_I greater than 1 indicates that the greater amount of labeled precursor incorporation is due not only to additive effects of C and Wm, but there exists a proliferative response other than the basal incorporation. The peaks of RNA and protein syntheses were shown to be 48 hours after initiation of culture while that of DNA, 72 hours. Bach and Voynow (1966) suggested a culture period of 7 days before labeling. However, this is close to impracticable in view of the fact that mouse lymphocytes are particularly difficult

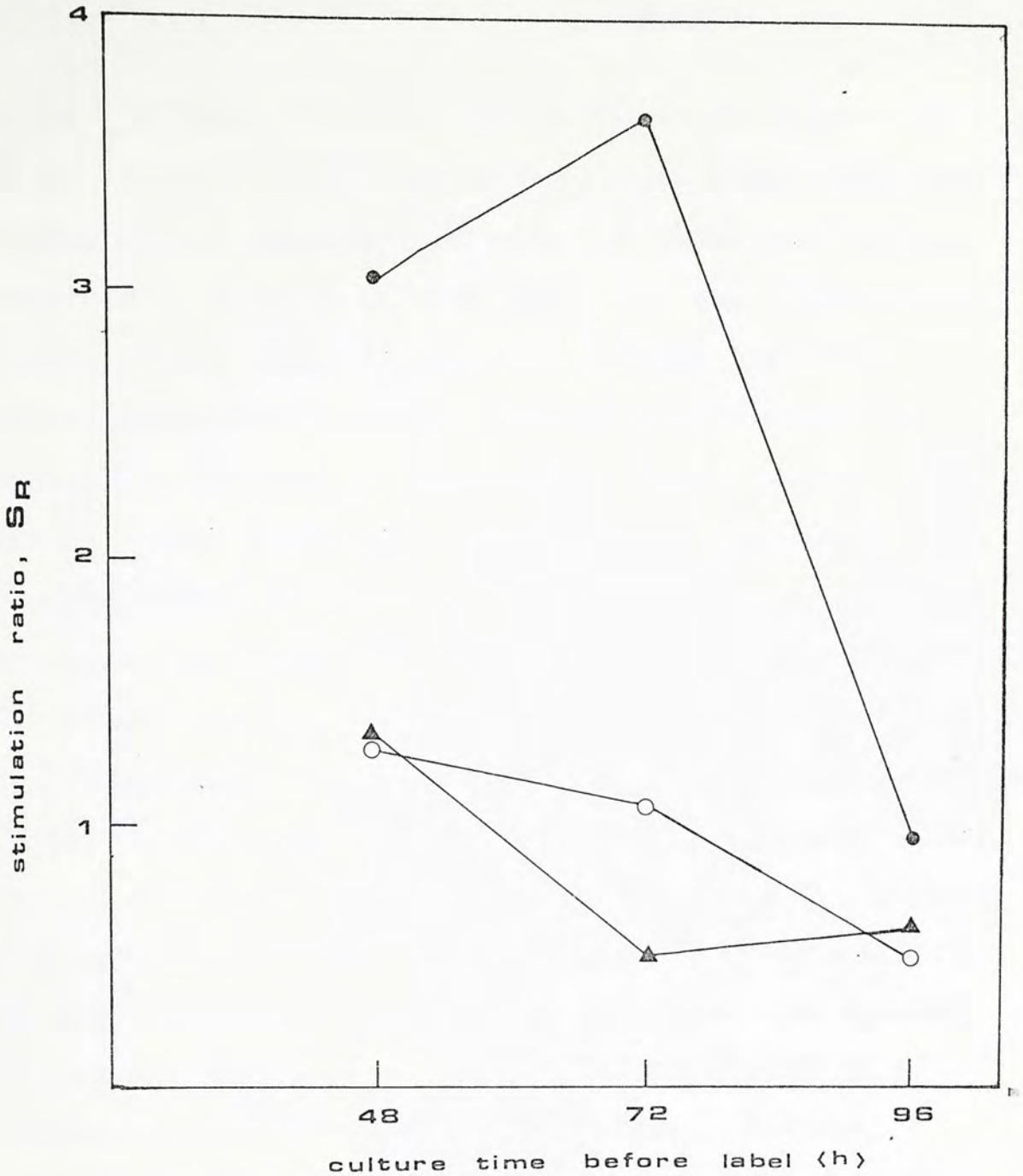


Figure 3.11 The kinetics of macromolecular syntheses in mixed lymphocyte reaction of CWm.

$$S_I = \frac{\text{cpm of CWm}}{\text{cpm of C + cpm of Wm}}$$

C = untreated CBA lymphocytes

Wm = mitomycin C treated WHT lymphocytes

Equal volumes of C and Wm cells (80 µl, 5 x 10^6/ml) were cultured for 48, 72 or 96 hours before labeling with ^{3}H-TdR (●), ^{3}H-UdR (▲) and ^{3}H-Leu (○). Cells were harvested 20 hours after labeling.

to maintain in vitro (Ling and Kay, 1975). They reported that of an initial 2 x 10^6 /ml mouse spleen cells cultured in RPMI1640 medium supplemented with 5 % fetal calf serum, approximately 30 % would be dead after one day, 65 % after 3 days and 75 % dead after 4 days. It follows that MLR quantitated after too lengthy a culture period would be erroneously low because of the extensive cell death. Hayry and Defendi (1970) also reported that under commonly used culture conditions, stimulation in mixed spleen cell cultures was of a much lower order with an ill-defined maximum on days 3 to 6. These data are consistent with the stimulation kinetics observed in the present study. The S_I values of less than unity at later times reflect the increasing cell death.

The effects of TCS on protein, RNA and DNA syntheses in MLR were shown in Figure 3.12. CWm cells were cultured with various concentrations of TCS. Cultures were labeled with ^{3}H-UdR and ^{3}H-Leu at 48 hours and with ^{3}H-TdR at 72 hours and harvested 20 hours after labeling. A dose dependent inhibition by TCS on MLR was observed. In all cases, a TCS concentration of 10 µg/ml or above reduced the S_I to less than unity. The steeply falling curve in fig. 3.12 once again manifests the greater susceptibility of DNA synthesis to inhibition by TCS as previously observed on ConA-induced lymphocyte transformation.

Apart from the clinically used immunosuppressants, a number of agents or factors have also been reported to exert inhibitory effect on MLR (Ling and Kay, 1975). The better

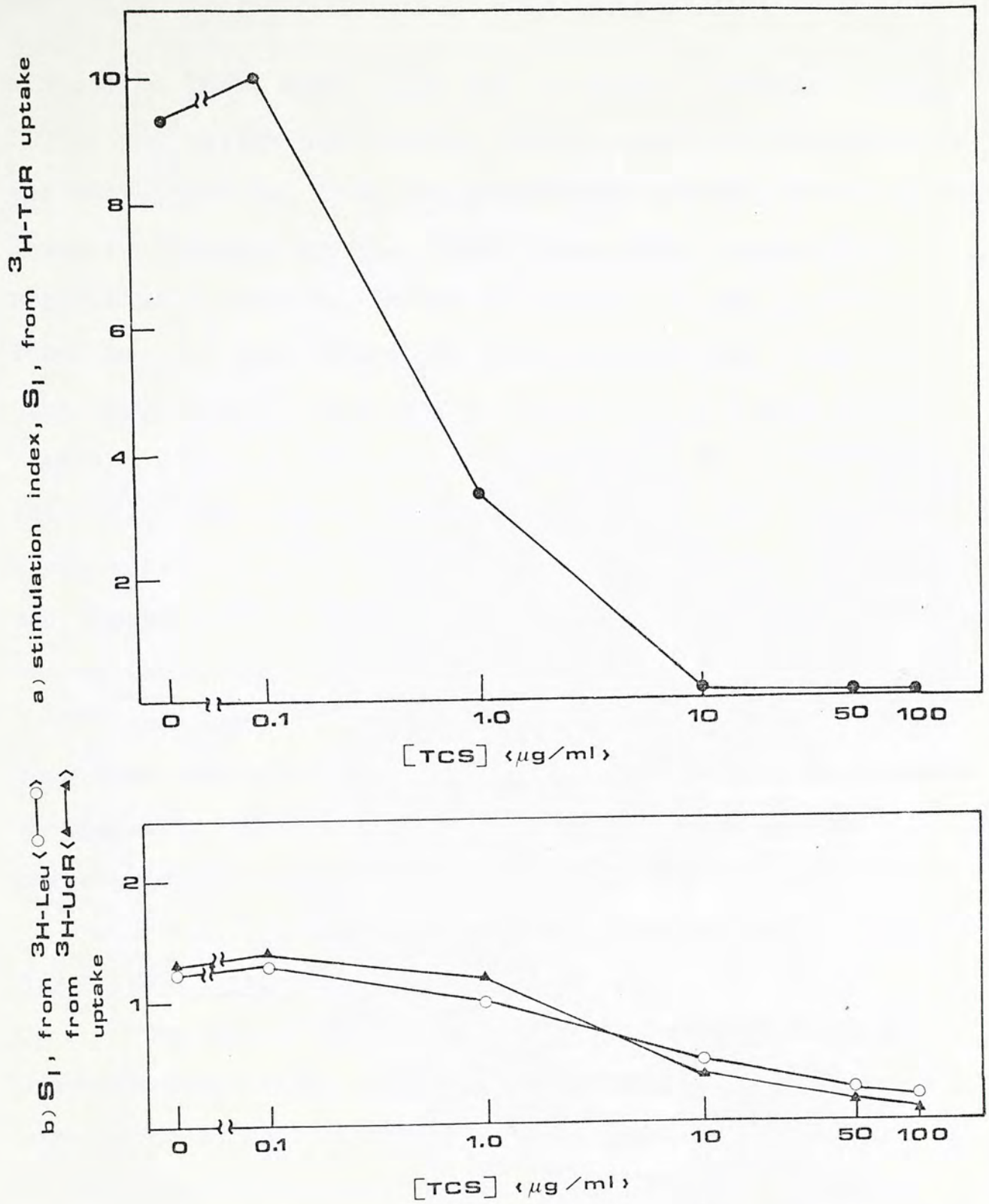


Figure 3.12 The inhibition of TCS on CWm MLR as measured by a) ^{3}H-TdR (●—●) uptake and b) ^{3}H-UdR (▲—▲) and ^{3}H-Leu (○—○) uptake

Culture was pulsed with ^{3}H-UdR and ^{3}H-Leu at 48 hours and ^{3}H-TdR, at 72 hours.

studied of these agents are HCG and α-FP . Teasdale et al. (1975) and Beling and Weksler (1974) reported independently the non-cytotoxic, specific inhibitory effects of HCG on MLR. However, Caldwell et al. (1975) found that inconsistent results were observed in the inhibition of HCG on MLR. They observed that crude HCG inhibited one-way human MLR from some donors, while other donors were not affected. Purified HCG also gave variable results. In cases where inhibition was present, no dose-dependency was observed. A controversial situation also exists for α-FP. Murgita and Tomasi (1975), using mouse amniotic fluid, observed a non-cytotoxic suppressive effect on mouse MLR. Sell et al. (1977) and Sheppard et al. (1977), using amniotic fluid or sera from hepatoma bearing mice and rats, failed to observe a consistent inhibitory effect of α-FP on MLR of the corresponding species.

3.2.4 Glycolytic activities

The effect of TCS on glucose uptake and lactate production by lymphocytes were investigated. Lymphocytes were cultured under different conditions for 48 hours, after which the glucose uptake and lactate production of cultures were determined within an incubation period of 2 hours at a saturating exogenous glucose concentration of 5 mM (Suter and Weidemann, 1975).

i) Glucose uptake

Glucose determination was based upon the following

coupled enzymatic reactions:

$$\text{Glucose} + 2\,H_2O + O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} \text{gluconic acid} + 2\,H_2O_2$$

$$H_2O_2 + \text{O-dianisidine} \xrightarrow{\text{peroxidase}} \text{oxidized O-dianisidine (brown)}$$

The intensity of the brown colour measured at 425-475 nm was proportional to the original glucose concentration.

$$\text{Glucose concentration mM} = \frac{Au}{As} \times 5.56$$

when Au = absorbance of unknown

As = absorbance of standard, which was 5.56 mM

$$\text{Glucose uptake} = C_t - C_o$$

where C_t = glucose concentration at time t

C_o = glucose concentration at time 0

Figure 3.13 presents the glucose uptake under various culture conditions. No significant detectable amount of glucose was observed to be taken up by the cell control culture. This may have been due to the fact that the metabolically rather inert resting lymphocytes suffice themselves with the endogenous glucose pool. Suter and Weidemann (1975), using rat spleen slices, found that even at near-saturating glucose concentration, endogenous substrates contribute more than 70 % of the oxidative fuel. On the other hand, cells cultured in the presence of con A showed a significant increase in the amount of glucose uptake. According to Parkes and Howell (1975), addition of PHA to lymphocyte cultures stimulates glucose

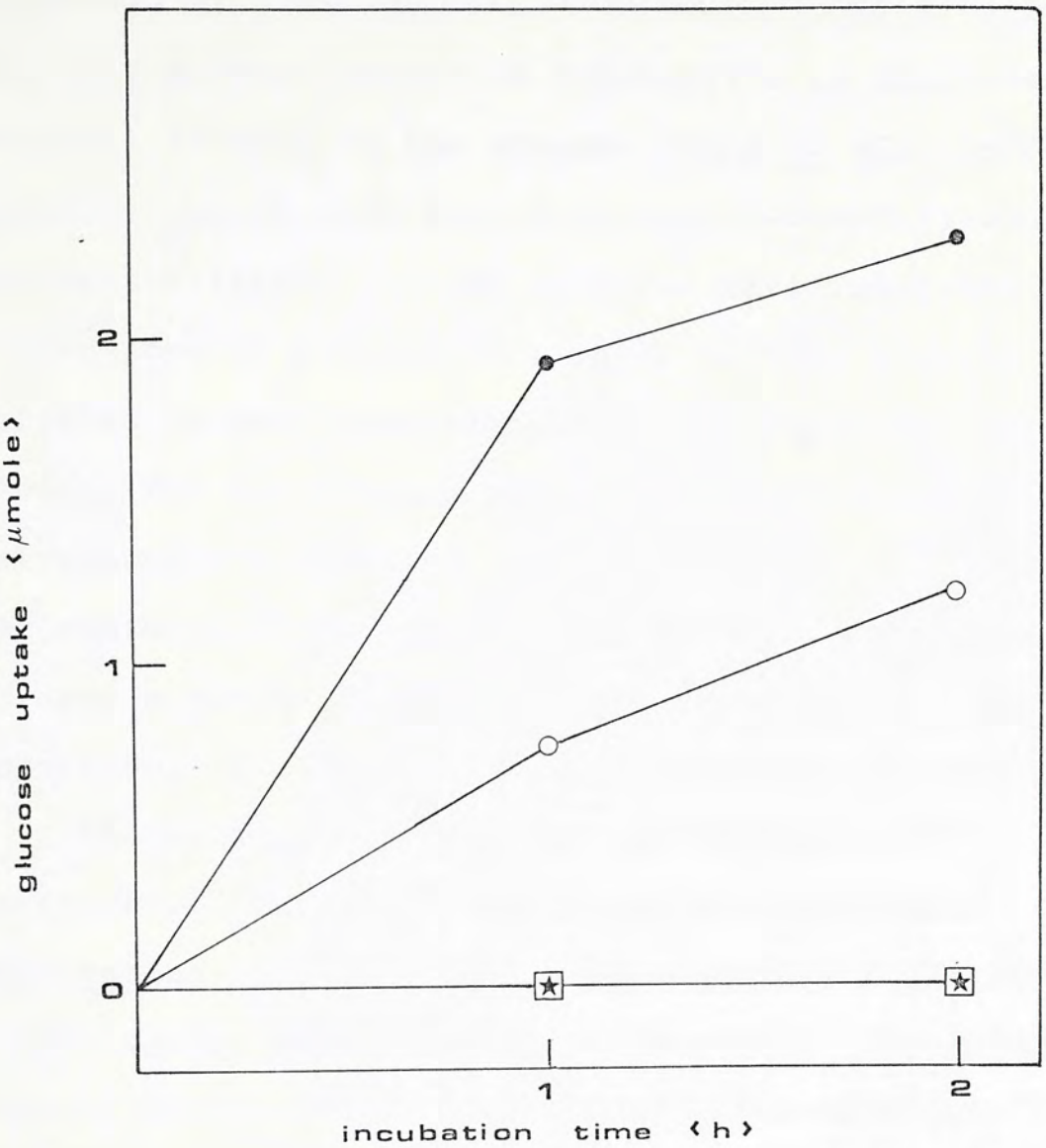


Figure 3.13 Inhibition of glucose uptake by TCS in con A-stimulated lymphocytes. Cell cultures were: control(□), con A(●), TCS(*), con A and TCS(○). Each point represents the number of μmole of glucose consumed by 9×10^7 viable cells after 48 hours of incubation, and then assayed during a 2-hour assay period.

consumption by these cells. Similarly it has also been shown that glucose uptake by lymphocytes is increased when con A is used as the mitogen (Wang et al., 1976a). Experiments which have been done with 3-O-methyl-glucose, a non-metabolizable glucose analogue have indicated that the transport of glucose is a rate-limiting step in its metabolism by rat thymus-lymphocytes. These all are evidences for the crucial role of glucose consumption for lymphocyte activation.

Cells cultured in the presence of both con A and TCS displayed a partial (~ 60 %) suppression on glucose consumption. The effective concentrations of con A and TCS in the culture were 3 μg/ml and 100 μg/ml, respectively. In ^{3}H-TdR uptake experiments, this concentration of TCS would have abrogated completely the blastogenic response induced by con A. The inhibitory action of TCS on other metabolic or non-metabolic pathways is implicated. Cells cultured with TCS, like the cell control, showed minimal glucose consumption, indicating basal glucose metabolism. There exists the possibility that suppression of glycolysis may be a "secondary" effect due to inhibition of lymphocyte proliferation by the presence of TCS in culture.

ii) Lactate production

Lactic acid was assayed basing on the following reversible reaction:

$$\text{Lactic acid} + \underset{(\text{low } A_{340})}{NAD^{+}} \longrightarrow \text{pyruvic acid} + \underset{(\text{high } A_{340})}{NADH}$$

In the presence of excess NAD^+, substantially all the lactic acid was converted to pyruvic acid which was trapped with hydrazine. The increased absorbance at 340 nm due to NADH formation was a measure of the lactic acid originally present in the sample.

$$\text{Lactate concentration (mM)} = \frac{Au}{As} \times 0.8889$$

where Au = absorbance of unknown

As = absorbance of standard, which was 0.8889 mM

Figure 3.14 depicts the rate of lactic acid production by lymphocytes cultured under various conditions for 48 hours. Con A treated cells showed an increase in lactate production of 3 fold over the control culture. Roos and Loos (1970) observed a doubled rate of lactate production within 15-30 minutes of PHA addition to human lymphocytes. Wang et al. (1976a) also reported a small but significant increase in lactate production for the first 20 hours of stimulation by con A. With the onset of DNA synthesis at approximately 20 hours, there was a sharp increase in the rate of lactic acid production. The present result is also consistent with the report of Pachman (1967) that lactate production rises to about 4 times the initial value after 24 hours.

Pachman (1967) also observed a general correlation of lactic acid production and glucose consumption to be in a ratio of 2:1. This reflects that the majority of the glucose utilized is converted to pyruvate or lactic acid.

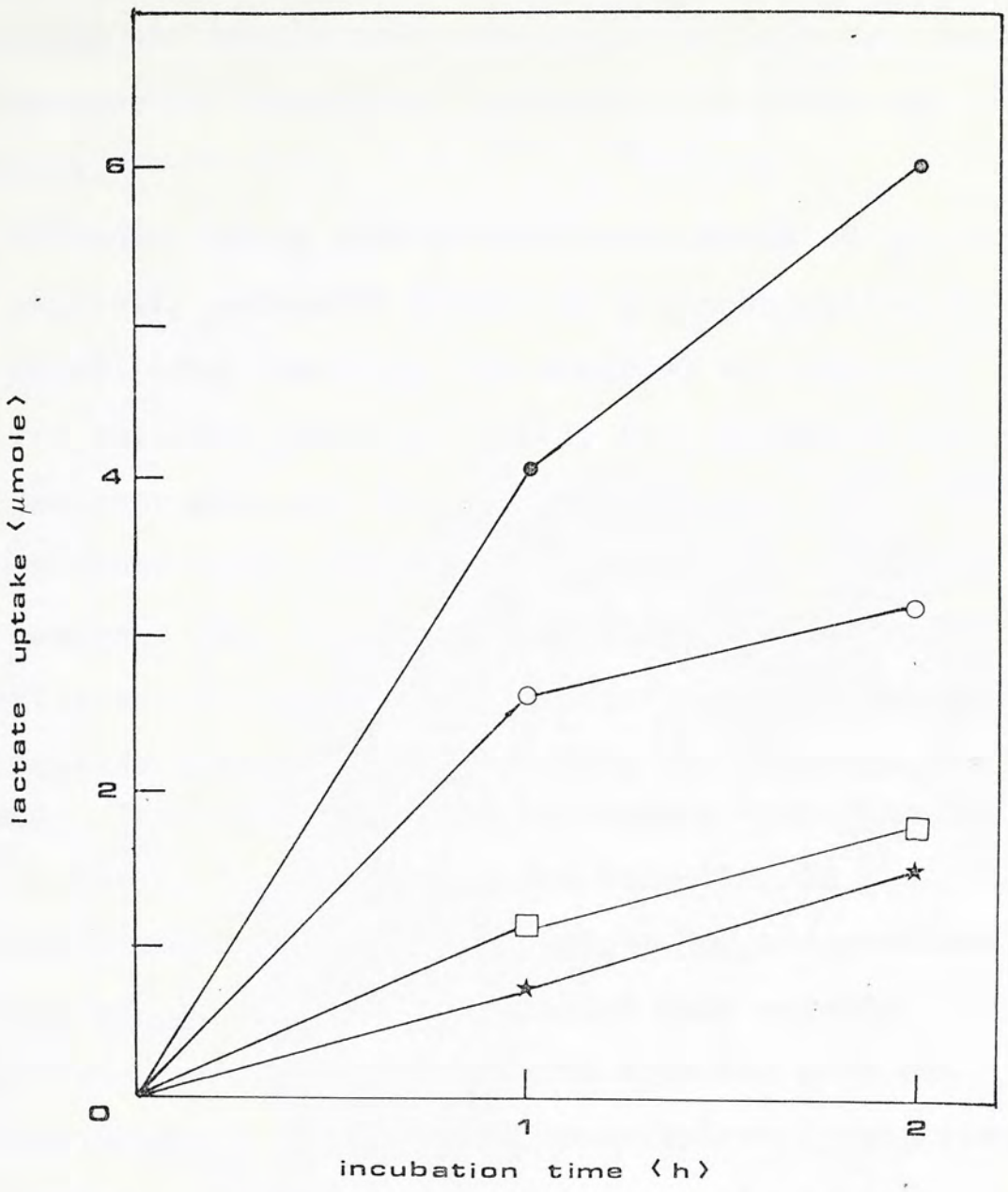


Figure 3.14 Inhibition of lactate production by TCS in con A-stimulated lymphocytes. Cell cultures were: control (□), con A (●), TCS (✱), con A and TCS (○). Each point represents the lactate produced by 9×10^7 viable cells after 48 hours of culture, and then assayed during a 2 hours assay period.

Comparing the lactic acid production and glucose uptake in the present experiment, a roughly 2:1 ratio can be obtained.

The enhanced lactic acid production stimulated by con A was partially abrogated by TCS at a concentration of 100 μg/ml. The reduction was about 40 %, which was smaller than the around 60 % reduction observed for glucose consumption. This may be explained by the fact that glucose consumption measurement is less sensitive and accurate than lactate production. The lack of sensitivity in glucose determination has been reported by Hedeskov and Esmann (1965) using the same assay method. That this reduction in lactate formation by TCS is one of the causes for the reduction in DNA synthesis observed is brought out by the observation of Wang et al. (1976a) They showed that aerobic glycolysis considerably increased together with DNA synthesis in con A stimulated mouse spleen lymphocytes. Futhermore, with the return of these cells to non-proliferative state, lactate production also fell. However, at 100 μg/ml of TCS, complete abrogation of DNA synthesis was observed while the inhibition on lactate production was only partial. Inhibitory effects of TCS on other processes are strongly implicated. A slight reduction (average 20 %) in basal lactate formation was observed in TCS treated lymphocytes. The inhibition on basal DNA synthesis is hence accounted for.

3.2.5 Oxygen consumption

The rate of oxygen consumption by splenocytes cultured under various conditions was measured polarographically by an oxygen electrode (YSI Model 50). The O_2 consumption rate was first investigated as a course of time of con A stimulation. The respiration rates of cells cultured in TCS, TCS + con A, con A and cell control were then measured at the time when specific increase in O_2 uptake rate was maximal.

Roos and Loos (1970) found that the Krebs cycle activity of human lymphocytes was increased within about 2 hours of PHA addition to cell cultures and it was estimated that as much as 85 % of the ATP synthesized by lymphocytes may be formed as a result of oxidative phosphorylation even though only 25-35 % of the glucose was oxidized completely while 60-70 % was converted to lactate (Roos and Loos, 1973). Thus proliferating lymphocytes undergo active aerobic glycolysis. Wang et al. (1976a)observed a 20 fold increase in aerobic glycolysis in human lymphocytes by 50-60 hours after con A stimulation and the increase occurred coincidentally with the increase in DNA synthesis but not with RNA and protein syntheses. They suggested that aerobic glycolysis in stimulated lymphocytes was related to DNA synthesis and was not due to either an increase in cell size or to cellular differentiation.

The O_2 consumption rates were first recorded as the percent decrease in O_2 saturation per minute. The solubility

of O_2 in distilled water at 37°C was taken as 200 μmole /l according to Estabrook (1967). The cell number used was corrected for from 0.875 x 10^7/ml to 1 x 10^7/ml to give rates of O_2 consumption in the unit of mμmole /10^7 cells /min. Table 3. 4 shows the rates of O_2 consumption of con A stimulated cultures and control cultures. An increase in rates of about 1.5 fold in stimulated culture was maintained throughout the time period studied. These observations are consistent with those of the above-mentioned workers.

The rates of O_2 consumption by variously treated cultures were investigated after 8 and 12 hours of incubation. Similar stimulated rates of O_2 uptake were again observed. Generally speaking, the consumption rates at 12 hours are higher than at 8 hours, reflecting an increasingly active aerobic respiration after con A stimulation. The effective concentration of TCS in the culture was 100 μg/ml, but the con A stimulated increase in O_2 consumption was only partially (21 % at 8 hours and 36 % at 12 hours) suppressed (Figure 3.15). A greater inhibition is anticipated after longer periods of culture with TCS. The intervals of 8 hours and 12 hours were chosen in view of the poor viability of lymphocytes cultured <u>in vitro</u> (see MLR, Section 3.2.3) and the reasonable stimulation over control values at these time intervals. The inhibitory effect of TCS on basal respiration rate was again observed.

Table 3.4 The rate of O_2 consumption by con A stimulated lymphocytes as a function of time. Cells (1×10^7/ml, 3.5 ml) were incubated with or without con A (3 µg/ml) for the indicated time periods. Oxygen consumption was measured by using an O_2 electrode and the rate expressed as mµmole / 10^7 cells / min.

Length of con A stimulation (hr)	O_2 consumption rate		Fold of stimulation
	Control	con A	
0	0.66	1.01	1.53
16	0.75	1.30	1.73
39	0.77	1.14	1.48
43	0.97	1.86	1.92

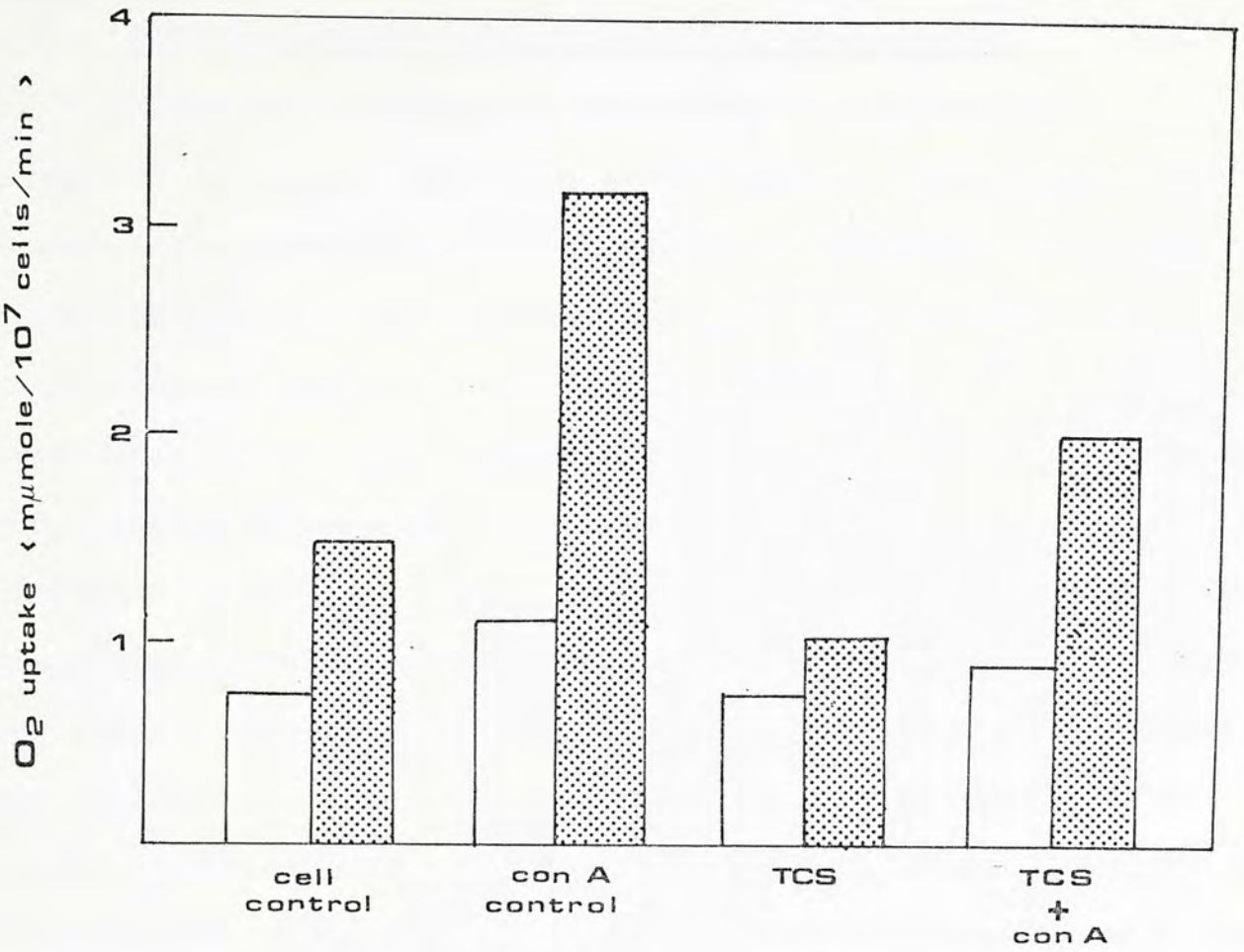


Figure 3.15 Inhibition of TCS on O_2 uptake of con A stimulated lymphocytes. Splenocytes (1×10^7 /ml, 3.5 ml) were cultured under various conditions for 8 □ and 12 ⊡ hours. Then O_2 uptake was measured by an O_2 electrode.

In Vivo Studies

3.2.6 In vivo TCS pretreatment on subsequent in vitro mitogen-induced lymphocyte transformation

The distribution of iodinated TCS after i.p. injection into mice has been investigated. Lau et al. (1980) reported the distribution of iodinated Trichosanthin injected intraperitoneally into 12-day pregnant mice. A large proportion of the radioactivity was recovered in the blood serum which serves as a carrier. However, radioactivity could still be detected in different organs even after extensive removal of blood by perfusion. Low ^{125}I was found associated with the ovary, uterus and mammary glands, whereas the kidney exhibited a high ^{125}I-radioactivity at all times, and followed by the liver and spleen. By contrast, ^{125}I-radioactivity declined between 12-48 hours after treatment in other organs, including the reproductive organs, blood stream and amniotic fluid. The persistently high ^{125}I in the kidney indicated that it was the major site of TCS elimination. Considerable sequestration of TCS by the spleen was indicated.

Mice were treated twice intraperitoneally with TCS at 0.2 mg/25 gm body weight in 0.2 ml of PBS 4 and 2 days before sacrificing for culture. Control animals received 0.2 ml of PBS. In this study, male mice were used to eliminate any effects that the abortifacient activity of TCS might have on the female lymphoid tissues. The splenocytes of these animals were subsequently isolated and subjected to

in vitro mitogenic stimulation by con A and LPS.

Fig. 3.16 and 3.17 show the mitogenic response of splenocytes to con A and LPS after *in vivo* TCS pretreatment. The response was presented as the stimulation ratio, S_R.

$$\text{And } S_R = \frac{\text{cpm of stimulated cells}}{\text{cpm of cell control}}$$

A S_R value of 1 indicates no stimulation.

This i.p. TCS treatment resulted in a reduction in the blastogenic responses of splenocytes to con A and LPS. The reduction in S_R was extensive, ranging from 6.1 to 19.1 fold at various doses of con A. On the other hand, the reduction in LPS stimulation was less significant, differing only by 1.3 fold. This difference in susceptibility might be a reflection of the anatomical arrangement of T-dependent and T-independent areas in the spleen. The T-dependent areas occupy a periarterial position in the white pulp, therefore comes earlier into contact with TCS in the circulation. The T-independent areas are found within the marginal zone and red pulp, which are more remote from the blood supply.

In this experiment, 3 doses of mitogen were used instead of using only the concentration that would normally elicit optimal stimulation. This was done to eliminate the possibility that any reduction in S_R value in the treated animal compared to the control may be the result of a shift in the mitogen dose-response profile in the treated animal rather than an inhibitory effect of TCS. It is observed here that both treated and control cells have similar

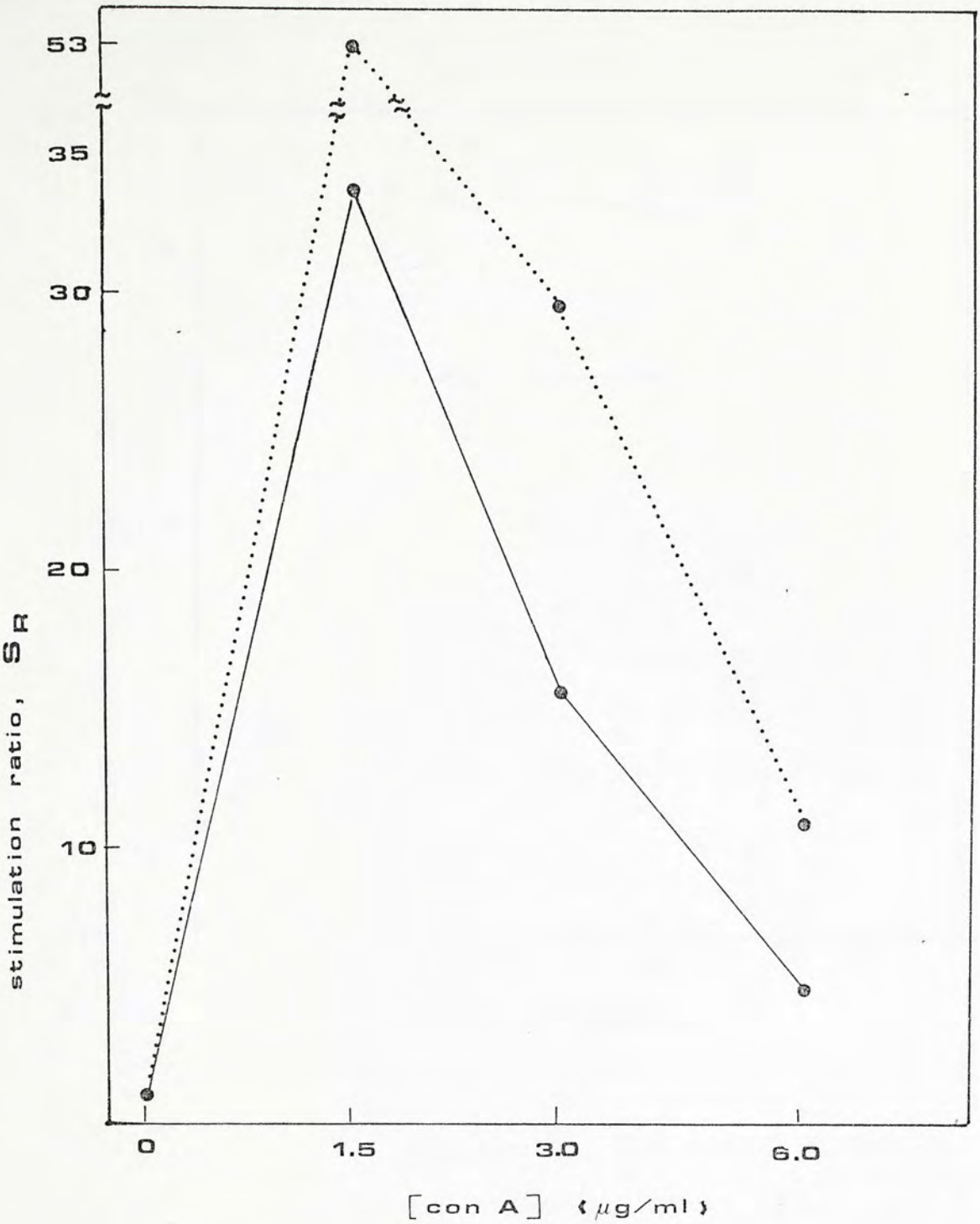


Figure 3.16 Effect of *in vivo* TCS pretreatment on subsequent stimulation by con A.

$$S_R = \frac{\text{cpm of stimulated culture}}{\text{cpm of corresponding cell control}}$$

●·····● control, animal i.p. PBS twice

●——● treated, animal i.p. TCS twice (0.2 mg/25 g body weight)

Splenocytes were isolated, cultured with various doses of con A for 48 hours, then labeled with ^{3}H-TdR and harvested 6 hours later.

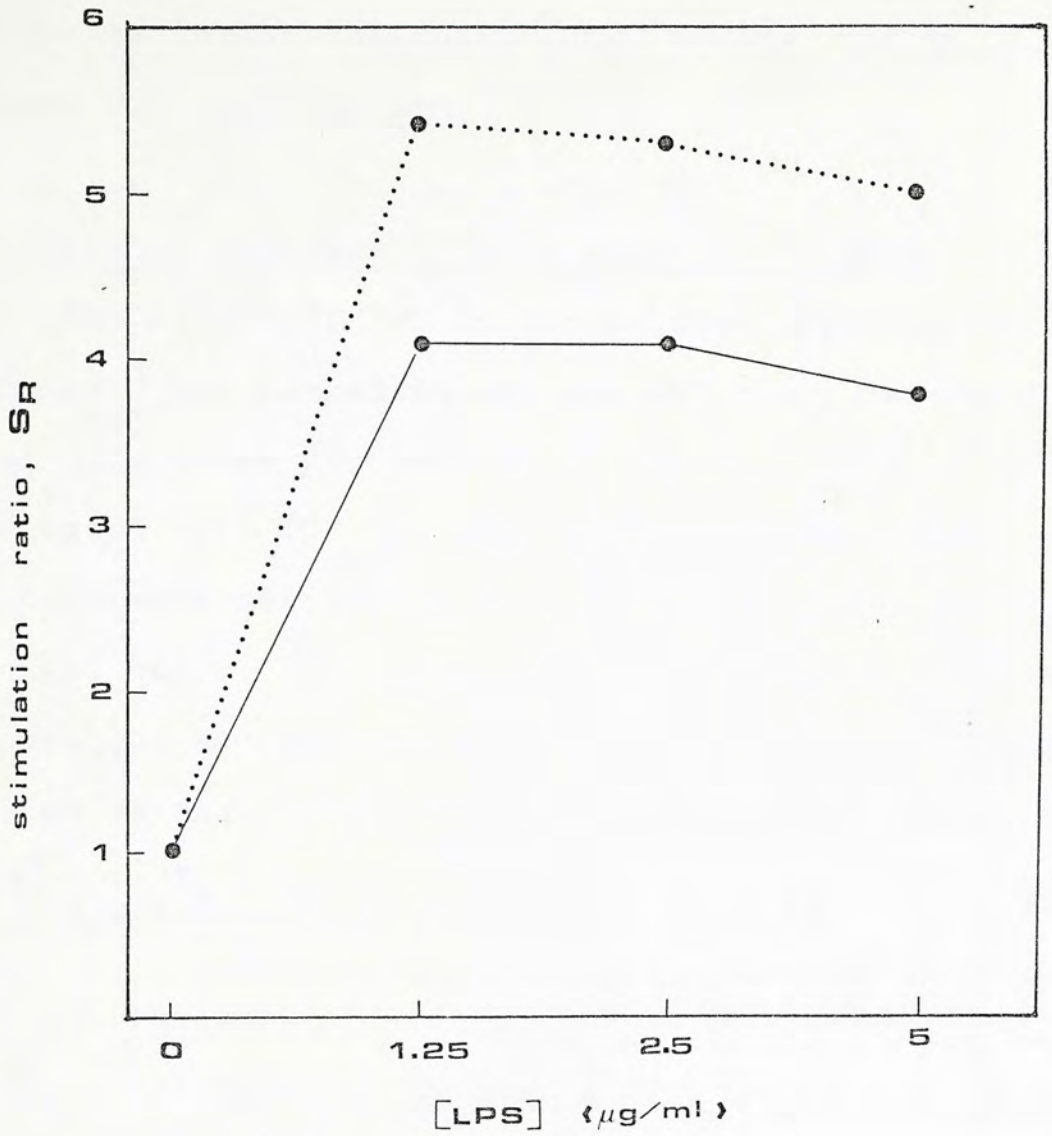


Figure 3.17 Effect of <u>in vivo</u> TCS pretreatment on subsequent stimulation by LPS.

$$S_R = \frac{\text{cpm of stimulated culture}}{\text{cpm of corresponding cell control}}$$

●·····● control, animal i.p. PBS twice

●——● treated, animal i.p. TCS twice (0.2 mg/25 g body weight)

Splenocytes were isolated, cultured with various doses of LPS for 48 hours, then labeled with ^{3}H-TdR and harvested 6 hours later.

optimal stimulation concentrations, namely, 1.5 μg/ml of con A and 2.5 μg/ml of LPS.

3.2.7 Delayed hypersensitivity reaction to SRBC

The effect of TCS on the delayed hypersensitivity (DH) to SRBC was tested in CBA and WHT mice. Animals were divided into three groups: E, C_1 and C_2. Mice in group E were treated with TCS two days before sensitization. Group C_1 animals were sensitized and challenged while group C_2 was challenged only. The increase in footpad thickness of the right, challenged over the left, unchallenged control was expressed as the percent specific swelling, P, where

$$P = \frac{T_R - T_L}{T_m} \times 100\ \%$$

where T_R = thickness of right footpad

T_L = thickness of left footpad

T_m = mean thickness of left and right footpad of all experimental animals before challenge

As shown in Table 3.5 , group C_1 animals in both CBA and WHT mice had a P value of 15 to 20 %. The maximal response was observed at 24 hours after challenge and declining thereafter albeit still maintained at considerable level at 48 and 72 hours. The severity of the reaction was different in WHT and CBA mice, with the former manifesting a greater reaction (22 % as compared with the 16 % of CBA mice), but declining more rapidly. These reaction kinetics and strain difference are consistent with the observations

Table 3.5 Inhibition by TCS of DH response to SRBC in

a) CBA mice

b) WHT mice

a) CBA mice

group	treatment	% specific swelling	
		24 hours	48 hours
C_1	sensitized and challenged	15.7	11.4
C_2	challenged only	2.4	0
E	TCS treated, sensitized and challenged	1.6	2.7

b) WHT mice

group	treatment	% specific swelling		
		24 hours	48 hours	72 hours
C_1	sensitized and challenged	22.0	11	5.7
C_2	challenged only	1.2	0	2.2
E	TCS treated, sensitized and challenged	3.8	0	0

of Mitsuoka et al. (1978). They reported that the time course of the DH reactivity after i.v. sensitization with 10^6 SRBC revealed the optimal sensitization-challenge interval to be 4 days. In the 4th day challenged mice, the DH reaction became apparent at about 12 hours, reached a peak at 24 hours and subsequently diminished, although the intensity was still considerable at 48 hours. They used 4 strains of mice in their experiments and observed a difference in the magnitude of the response under the same experimental conditions.

Within experimental error, animals in group C_2 showed little DH response. This is expected since they have not been sensitized. At the time of challenge, it was only the first time that the mice were exposed to the antigen, SRBC. Since the DH reaction involves the recruitment of naive macrophages and lymphocytes by presensitized cells to the site of antigen challenge, the absence of sensitization would not elicit any response. This absence of response also ruled out the possibility that the response observed in group C_1 animals were due to non-specific inflammation caused by the subcutaneous challenge of SRBC.

The percent specific swelling of mice in group E was small and close to values of group C_2. Based on the above discussion, this could only be attributed to a suppressive effect of TCS on the DH to SRBC. In this experiment, TCS was administered before sensitization. It remains to be elucidated whether the suppressive effect of TCS was exerted

either on the sensitization (afferent) phase or the recruitment (efferent) phase or both. The inhibition by interferon (IF) on DH in the mouse to picryl chloride and SRBC have been studied by Maeyer et al. (1975) and Maeyer-Guignard et al. (1975). They reported that in mice sensitized to picryl chloride or SRBC, expression of DH was significantly inhibited if the animals were treated with interferon or interferon inducing viruses (Sendai virus and Newcastle Disease Virus, NDV) on the day before or the day of challenge with the antigen. The IF inhibition on the afferent phase (sensitization) was proven later by the administration of IF or viruses 24 hours before sensitization with the antigen. Thus the inhibitory effect of IF acts both on the afferent and efferent phases of the DH response. Whether the characteristics of TCS inhibition on DH response is similar or related to the IF inhibition warrants further investigation.

3.3 Mechanism of Action of Trichosanthin on con A-induced Lymphocyte Transformation

Mitogenic activation of lymphocytes can be divided into 3 stages. The first step in mitogenesis is the binding of the mitogen to specific receptors on the plasma membrane, followed by the activation mechanism, at present largely controversial, which transmits the signal of binding to the inner cell machinery. A sequence of biochemical events is then turned on to effect cell proliferation.

Inhibitors of lymphocyte transformation may act on one or all of these stages. The recognition of mitogen, the early steps of activation or the cell division itself may all be susceptible. In the experiments to be described, the influence of TCS on the binding of ^{125}I-con A to lymphocytes, the effects of exposing lymphocytes to TCS before and after con A stimulation, and the blast cell transformation were investigated. It is hoped that these experiments would shed some light on the mechanisms by which TCS exerts its inhibitory effects on the con A-induced lymphocyte transformation.

3.3.1 Binding of ^{125}I - con A to lymphocytes

Concanavalin A was iodinated and specific activity determined to be 0.04 mCi/mg protein. The binding of ^{125}I-con A by mouse splenocytes in the presence of various concentrations of TCS was investigated. Results are shown in Table 3.6 . The binding ratio R_b represents the fraction of the added radioactivity that was bound after extensive washing.

$$R_b = \frac{B-b}{A-b}$$

where B = cpm bound

A = cpm added

b = background

The R_b fluctuated between 0.35 and 0.53. No dose dependent reduction in the binding of ^{125}I-con A was observed. The low R_b obtained in the presence of cold con A confirmed that

Table 3.6 Binding of ^{125}I-con A by CBA murine splenocytes in the presence of TCS. Splenocytes (0.8×10^6) were incubated with 25,000 rpm of ^{125}I-con A and various doses of TCS. After 1 hour of incubation, cells were washed thrice. The radioactivity remaining bound was counted. Results are expressed as the binding ratio, R_b.

$$R_b = \frac{B-b}{A-b}$$

where B = cpm bound
A = total cpm added
b = background

Each value is the mean ± S.D. of triplicate samples.

(TCS) µg/ml	Mean R_b ± S.D.
200	0.429 ± 0.13
100	0.393 ± 0.04
50	0.386 ± 0.19
10	0.45 ± 0.24
1	0.399 ± 0.02
0.1	0.35 ± 0.06
0.01	0.531 ± 0.06
cell control	0.487 ± 0.09
☆con A control	0.098 ± 0.03

☆: cold con A was added

the iodinated moiety was con A and not any other molecules that were capable of binding to lymphocytes. Thus TCS did not seem to exert its inhibitory effect through interfering with the binding of con A to its specific receptor on lymphocyte surface. The similar R_b values of the cell control and TCS added assays ruled out possible steric interaction between TCS and con A, which would then be unavailable for binding.

3.3.2 Time course of inhibition

i) TCS pretreatment

Splenocytes were pretreated with TCS for various periods of time. Con A was added to all cultures at the 24th hour. Incubation was carried on for 48 hours more before labeling and harvesting. Control cultures in which PBS was added in place of TCS were done to eliminate any possible impairing effect of the act of addition might have on the culture. Results shown in Table 3.7 show that the longer the cells were exposed to TCS before stimulation, the greater would be the inhibition. The same phenomenon had been observed by Contractor and Davies (1973), Han (1974) and Han (1975) for the inhibition of HCG on lymphocyte mitogenesis. They showed that inhibition was greater when the lymphocytes were preincubated with the hormone one day or even one hour before

Table 3.7 Effect of TCS pretreatment on subsequent blastogenic response of splenocytes to con A. Cells were cultured and TCS added at various intervals. PBS was added to control cultures. At 24 hours after initiation of culture, con A was added to all cultures, which were then incubated for 48 hours more before labeling.

$$S_R = \frac{\text{cpm of TCS treated culture}}{\text{cpm of control culture}}$$

Length of TCS pretreatment (h)	Control cpm ± S.D.	TCS treated cpm ± S.D.	S_R
12	47036 ± 1736	1906 ± 192	0.04
18	51217 ± 5488	903 ± 219	0.02
20	49896 ± 2038	704 ± 149	0.01
22	49264 ± 1147	247 ± 17	0.005
24	49173 ± 5040	568 ± 56	0.01

PHA addition. Han (1975) speculated that HCG might compete for the PHA-binding sites of the lymphocytes. Since HCG has a carbohydrate portion which is 31.3 % of the glycoprotein by weight, it is not impossible that HCG may interact with the PHA receptor sites. However, no such explanation can be offered for TCS, which has been shown to be a pure protein containing no carbohydrate component. And it has been verified that TCS would not interfere with binding of con A to their receptor sites. Nonetheless, the possibility that prolonged contact with TCS would render the con A receptors reactive to TCS has not been excluded.

ii) Con A pretreatment

Trichosanthin was added to splenocyte cultures preincubated with con A for various lengths of time. Results shown in Figure 3.18 indicate that TCS added after 24 or more hours of con A prestimulation caused no inhibition on the blastogenic response. This is in accordance with the findings of Gunther et al. (1974) about the committment of lymphocytes to blastogenesis. They introduced α-MM into cultures of mouse spleen cells at various times after the addition of con A and found a gradual decrease in its capacity to inhibit the con A-stimulated incorporation of ^{3}H-TdR. Addition of the saccharide 20 hours after exposure of the cells to

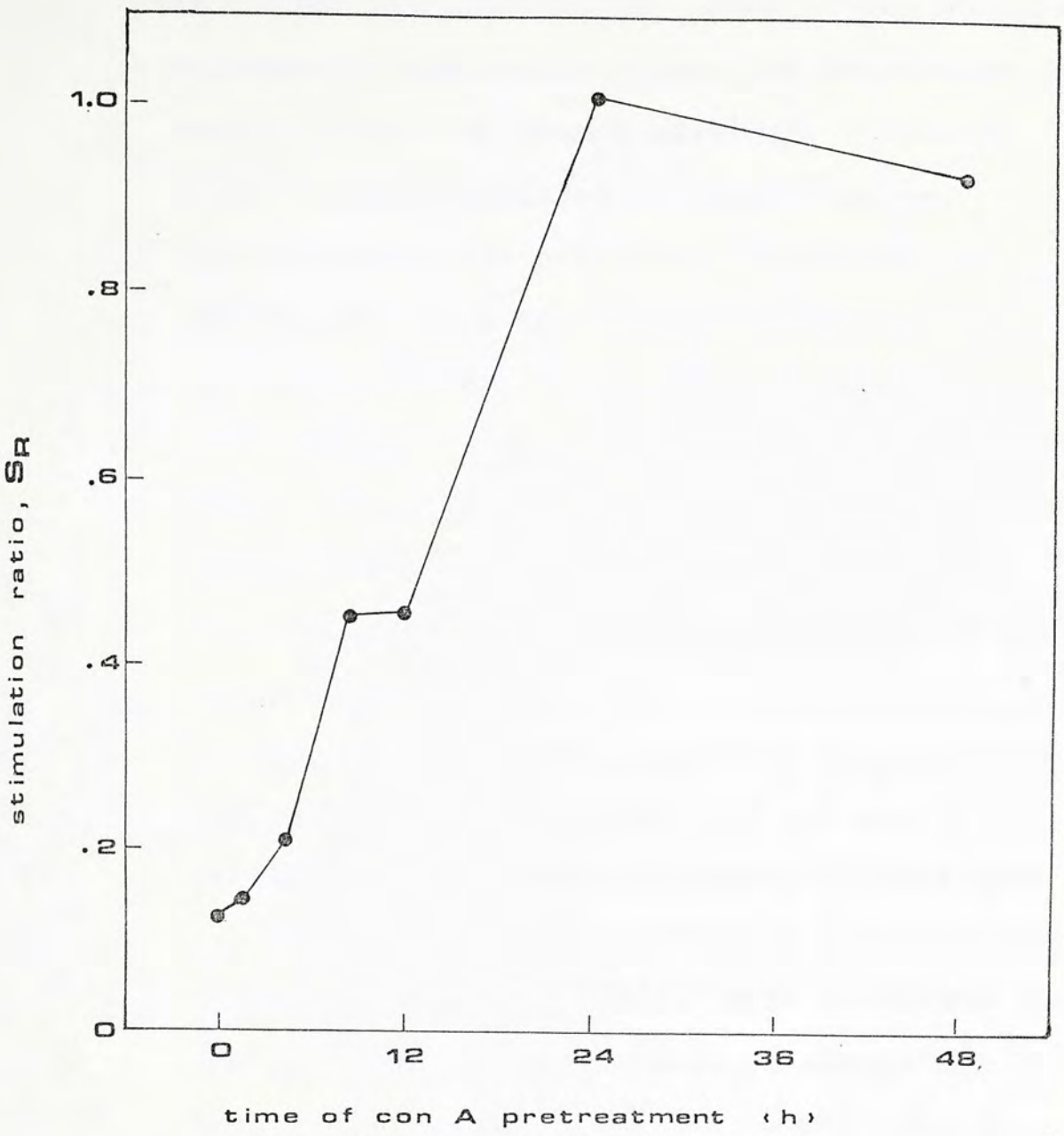


Figure 3.18 Effect of TCS on blastogenic response of splenocytes pretreated with con A for various periods of time. Splenocytes were cultured with con A (3 µg/ml) at the start of experiment. TCS (100 µg/ml) was added at times indicated and mixed well. PBS was added to control cultures. Cultures were pulsed with ^{3}H-TdR at 48 hours for 6 hours, and harvested.

$$S_R = \frac{\text{cpm of TCS added culture}}{\text{cpm of PBS added culture}}$$

con A had no effect on the level of the cellular response to the lectin. Thus TCS is similar to α-MM in that both cannot exert any effect on cells already committed to proliferation.

iii) Pretreatment with con A and Trichosanthin

Splenocytes were incubated with TCS and con A. At various times, aliquots of splenocytes were centrifuged and washed thrice to remove TCS and con A. Cells were resuspended and cultured in microtiter plates in two ways: one was the control where medium was added, the other had con A added.

Results in Table 3.8 show that irrespective of the length of simultaneous TCS and con A pretreatment, subsequent removal of both and cells recultured, the amounts of radioactivity incorporated were similar. This confirmed that 100 μg/ml of TCS was capable of abrogating completely the con A effect. Otherwise, an increasing amount of radioactivity should be obtained with increasing time of con A exposure. Results of cultures reconstituted with con A suggest that removal of TCS within the first 12 hours would allow partial stimulation, the magnitude of which was indirectly proportional to the length of pretreatment.

If TCS was removed after more than 12 hours of

Table 3.8 Effect of simultaneous TCS and con A pretreatment on subsequent Blastogenic response to con A. Splenocytes were bulk cultured in the presence of TCS and con A for various periods of time. Cells were then washed and recultured in either con A or PBS (control).

$$S_R = \frac{\text{cpm of con A reconstituted culture}}{\text{cpm of control culture}}$$

length of pretreatment(h)	control cpm$\pm$S.D.	+ con A cpm$\pm$S.D.	S_R
0	2226$\pm$165	53856$\pm$4470	24.2
4	3766$\pm$695	23584$\pm$3759	6.26
6	5206$\pm$1112	15526$\pm$2209	2.98
8	1946$\pm$349	5052$\pm$457	2.59
12	3503$\pm$483	3405$\pm$169	0.97
24	3104$\pm$430	2984$\pm$238	0.95
32	2622$\pm$183	2575$\pm$104	0.98
48	640$\pm$64	539$\pm$25	0.84
no treatment	1588$\pm$222	45618$\pm$5557	28.7

treatment, the stimulation would return to the unstimulated level. These results are shown in Figure 3.19 The stimulation indexes fell from 24.2 at zero hour to around unity at 12 hours of TCS and con A pretreatment. These results imply that TCS need not be present during the whole incubation to exert its inhibitory effect. Its presence during the first 12 hours was sufficient to bring about complete inhibition of con A-induced lymphocyte blastogenesis.

3.3.3 Blast cell transformation

Resting lymphocytes have very sparse, finely granular cytoplasm with few organelles consisting of several large mitochondria, some scattered ribosomes, a poorly developed Golgi apparatus and a few pinocytic vesicles. The nuclei contain heavy, dense clumps of heterochromatin and a small amount of euchromatin. Nucleoli are very small and are not readily seen. Transformed lymphocytes have a large number of ribosomes, a highly developed Golgi apparatus and increased numbers of large mitochondria. In the nucleus, heterochromatin is replaced with less dense euchromatin accompanied by large nucleoli.

When lymphocytes are stained with the Leishman stain, the nuclear material is stained rosy-purple and the cytoplasm, blue. In Figure 3.20a are shown normal, resting lymphocytes consisting of a large, round purple nucleus and a thin rim of

Figure 3.19 Effect of simultaneous pretreatment with TCS and con A on subsequent con A stimulation. Splenocytes were bulk cultured simultaneously with TCS (100 μg/ml) and con A (3 μg/ml). At the time intervals indicated, aliquots of cells were taken out and washed thrice to remove both TCS and con A. Cells were then recultured either in con A or PBS. Cultures were pulsed with ^{3}H-TdR at 48 hours for 6 hours, then harvested.

$$S_R = \frac{\text{cpm of washed cells recultured in con A}}{\text{cpm of washed cells recultured in PBS}}$$

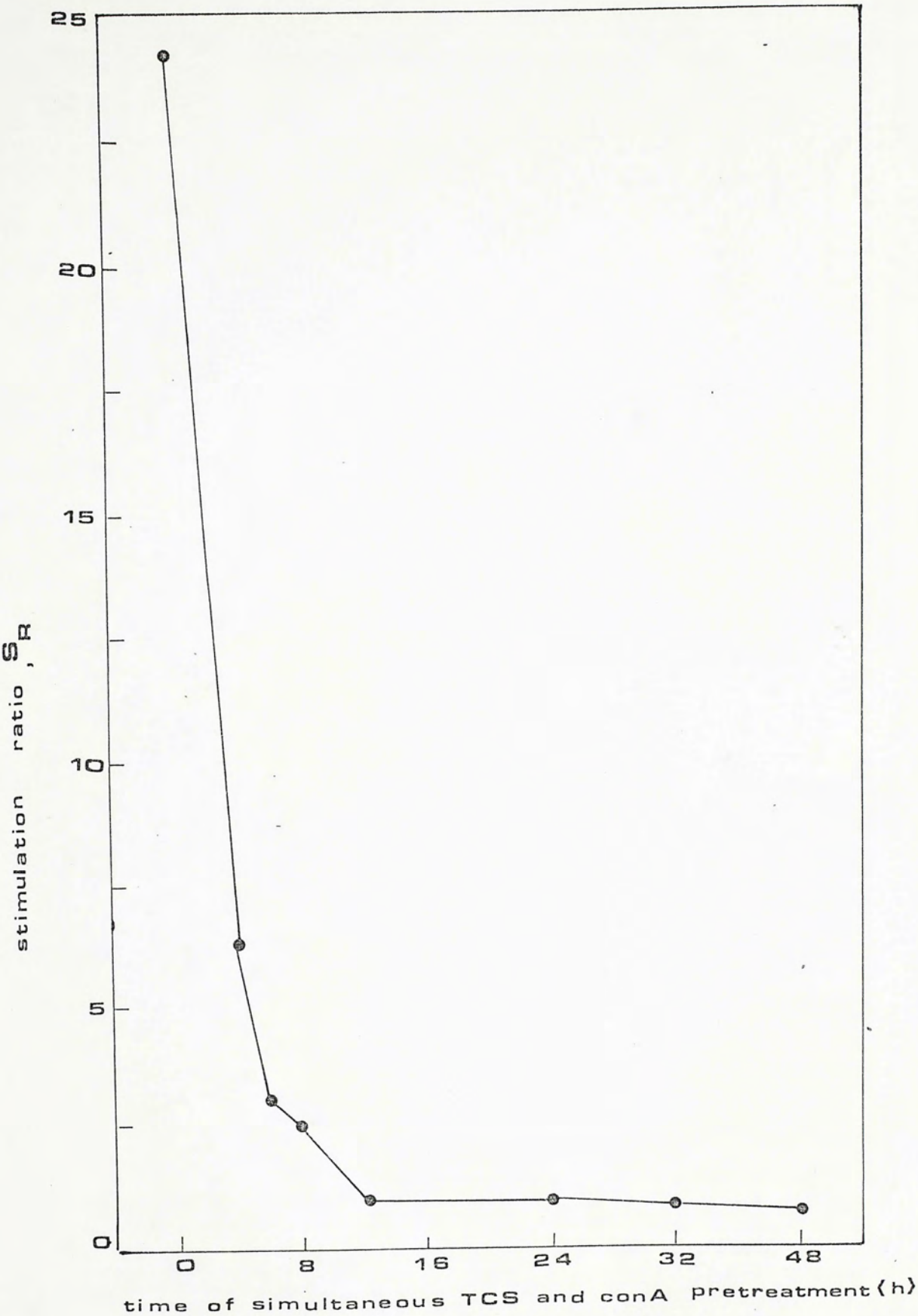

25
20
15
10
5
0
stimulation ratio, S_R
0
8
16
24
32
48
time of simultaneous TCS and conA pretreatment (h)

Figure 3.19

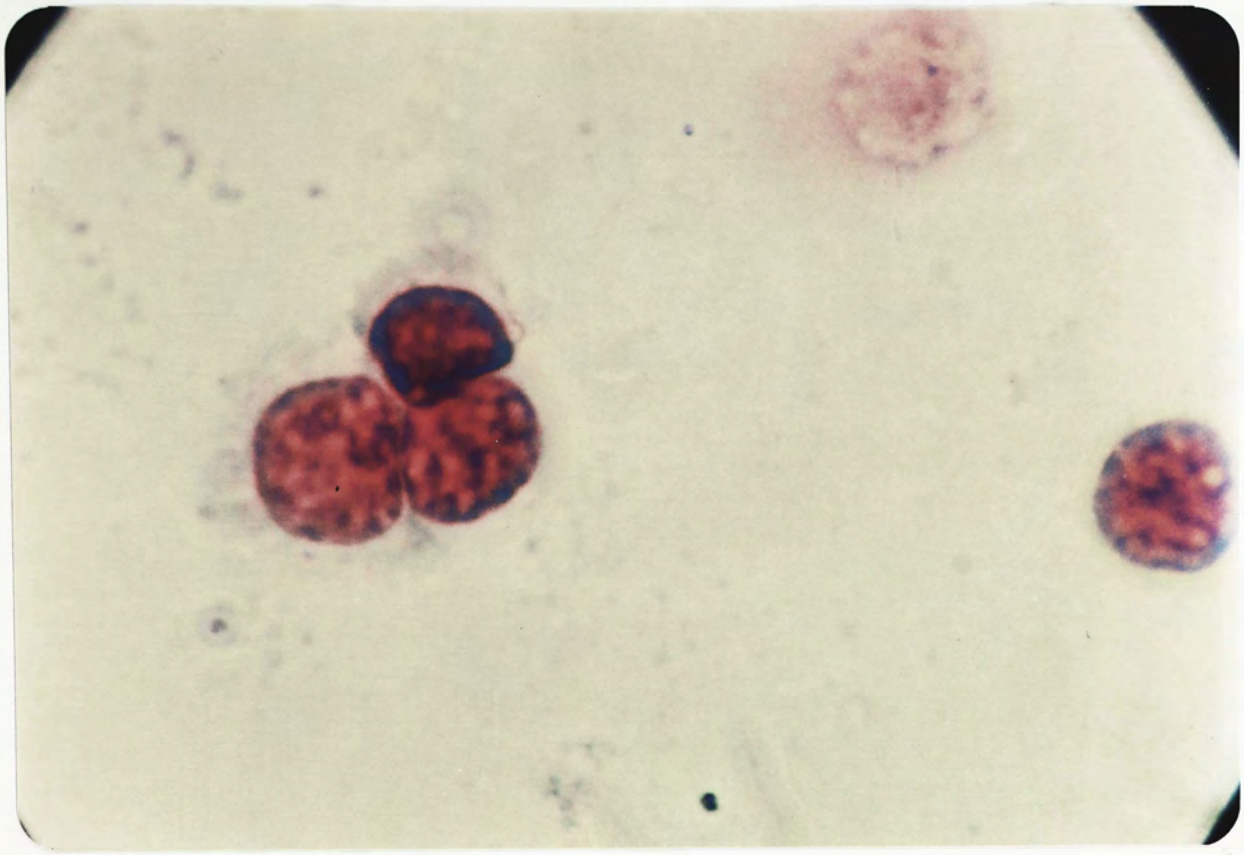
Figure 3.20 Lymphocytes Leishman-stained after 48 hours of culture with

a) no additive

b) con A (3 µg/ml)

Cell smears were prepared by a cytocentrifuge and then stained.

a)



b)

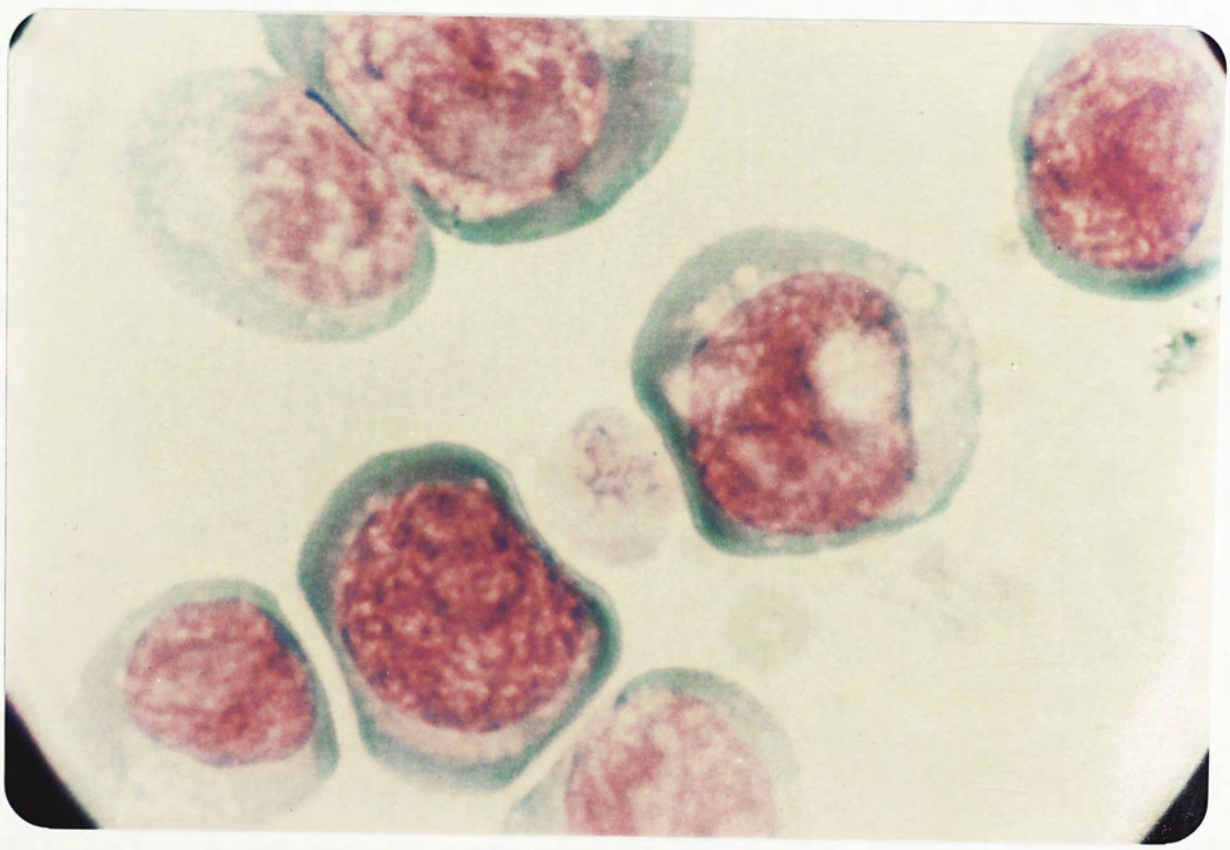


Figure 3 . 20

blue cytoplasm. In Figure 3.20b are shown con A-transformed mouse splenocytes. These cells have a large and slightly indented nucleus with less dense nuclear material. The cytoplasm is more prominent and vesicles are visible. The diameter of a small lymphocyte is around 10 μm while transformed cells may have a diameter of 30 μm.

Results of TCS on blast transformation by con A were shown in Figure 3.21 Lymphocytes are cultured in the presence of con A and TCS for 48 hours, and then Leishman stained. The number of small and blast lymphocytes were counted. Results are expressed as percentage blast, B, where

$$B = \frac{\text{number of blast cell}}{\text{number of total cell}} \times 100\ \%$$

Figure 3.22 shows the presence of both a small resting lymphocyte and a blast cell in the culture. The cell seen in the upper left corner is a blast cell with prominent vacuoles. The cell seen in the lower margin of Figure 3.22 is a small lymphocyte with a large nucleus and scanty cytoplasm. Figure 3.21 shows the percentage blast under different culture conditions. Each result represents a counting of at least 250 cells. The untreated cell control also shows 11 % blast transformation. This is expected because lymphocytes have a continuum of sizes. The con A treated cells showed 5.5 fold increase in the percent of blast cells over the control culture. The percentage of blast cells in TCS treated cells was below that of the cell control, which is in accordance with the previous observation that TCS by itself would inhibit con A-induced proliferation.

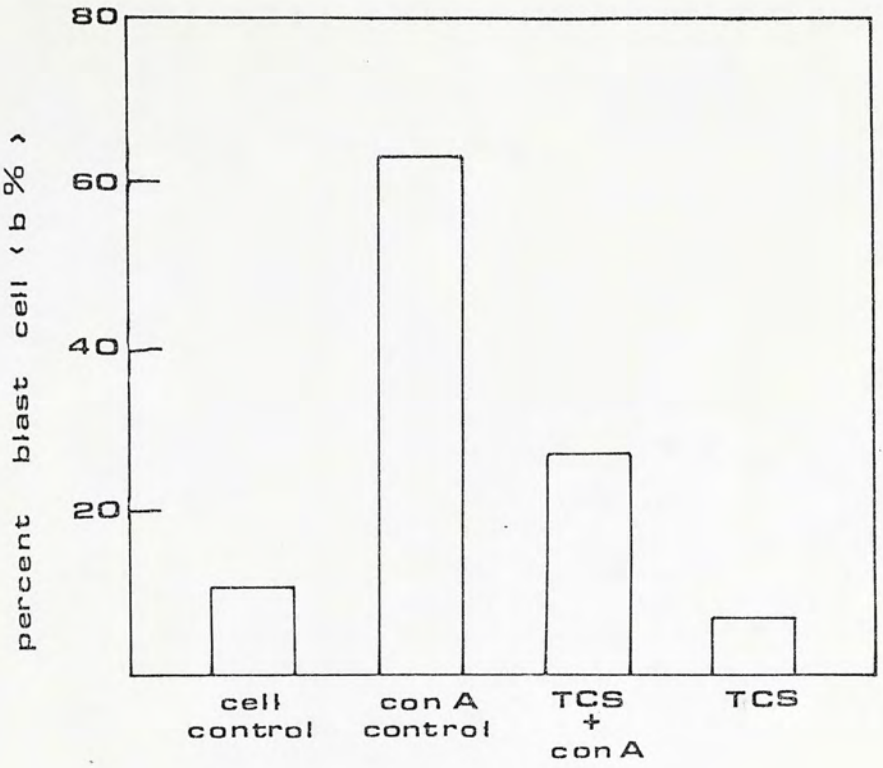


Figure 3.21 Inhibition of TCS on blast cell transformation from con A - stimulated mouse splenocytes. Cells were cultured in the presence or absence of TCS (100 μg /ml) and con A (3 μg /ml) for 48 hours. Cell smear of these cultures were prepared by a cytocentrifuge. The cell smear was Leishman - stained. Small and blast cells were counted under the microscope. Each bar represents a count of at least 250 cells.

$$\text{percent blast, } b\ \% = \frac{\text{number of blast cells}}{\text{total number of cells}} \times 100\%$$

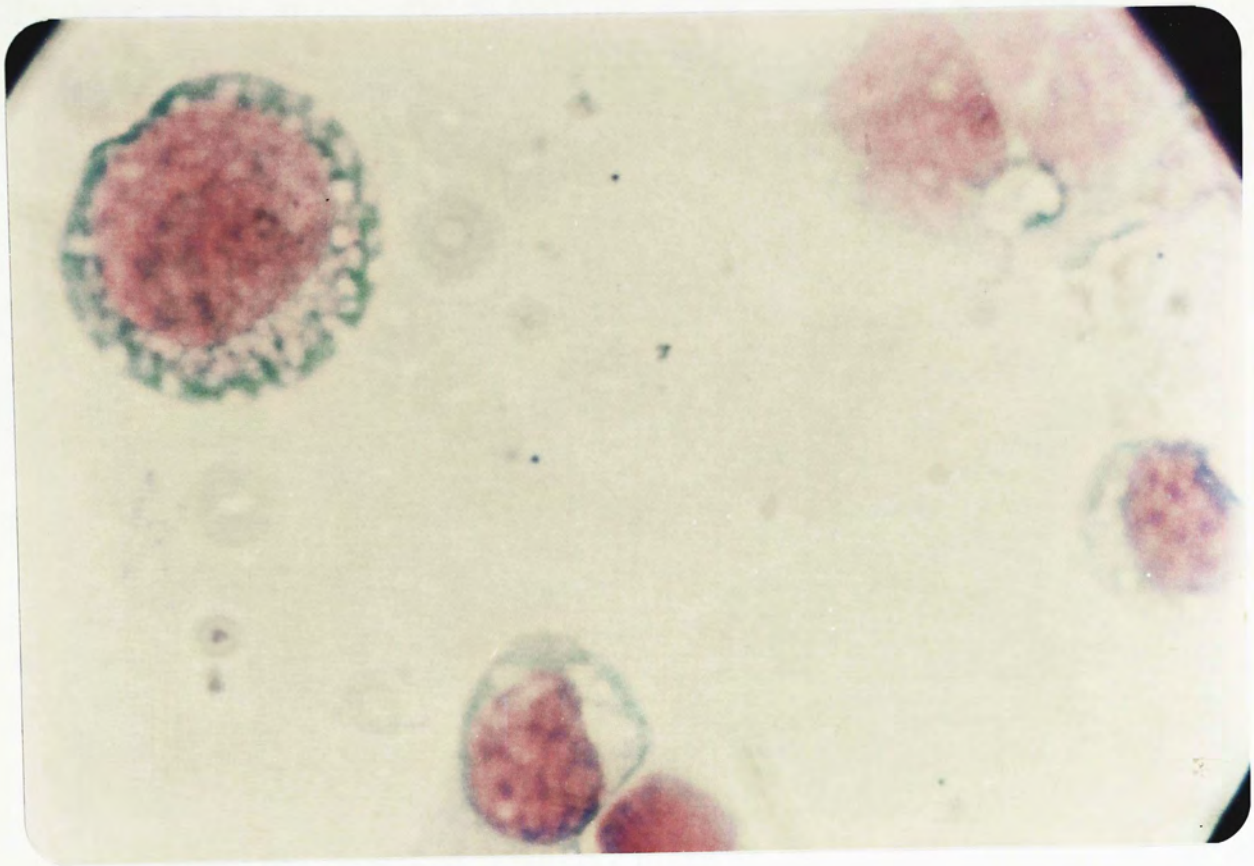


Figure 3.22 Small and blast lymphocyte in cultures with con A and TCS. Splenocytes were incubated with con A (3 μg/ml) and TCS (100 μg/ml) for 48 hours. Cell smears were then prepared on a slide by a cytocentrifuge. The cell smear was then Leishman stained.

It has been shown (Wedner and Parker, 1976) that in human peripheral lymphocyte incubated with PHA for 6 days or longer, as many as 70-90 % of the lymphocytes will assume a blast-like appearance. However, the number of cells which ultimately go into mitosis is relatively small, in some experiments as low as 3 %. Thus, this illustrates that blast cell transformation may not be synonymous with eventual cell division. Therefore, although there are blast cells seen in cultures containing TCS and Con A, these blast cells may not go on to mitosis and thus complete suppression of DNA synthesis can still be observed when compared to controls without TCS.

These results indicate that TCS inhibits lymphocyte transformation partly by reducing the number of cells that was committed to transformation rather than reducing the level of activation in the whole population. Parallel results have been reported by Gunther et al. (1974). They found that when con A transformation was inhibited by addition of α-MM, the percentage of blast cells and the percentage of ^{3}H-TdR labeled blast cells increased in parallel with the total radioactive thymidine incorporated while the average number of autoradiographic grains per labeled blast cell was relatively constant. The present observation supports the suggestion of Gunther et al. (1974) that the number of cells stimulated by con A increases with longer exposure to the lectin. Once a cell has become committed to mitogenesis, then DNA is synthesized at a rate independent of the amount of time the cell was exposed to con A.

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